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TITLE: Signal Transduction Pathway in Maspin-induced Tumor
        Suppression of Prostate Cancer

PRINCIPAL INVESTIGATOR: Karl X. Chai, Ph.D.

CONTRACTING ORGANIZATION: University of Central Florida
                           Orlando, Florida 32816

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
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6. AUTHOR(S) Karl X. Chai, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Central Florida Orlando, Florida 32816 E-Mail: kxchai@mail.ucf.edu			**8. PERFORMING ORGANIZATION REPORT NUMBER**
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13. ABSTRACT (Maximum 200 Words)

The purpose was to identify a maspin receptor. Prostasin and hepsin serine proteases were examined as candidates. Prostasin down-regulation observed in high-grade prostate cancer eliminated this protease as a candidate. Prostasin expression is absent in highly invasive DU-145 and PC-3 cells, while expressed in normal human prostate epithelial cells and the non-invasive LNCaP cells. A forced re-expression of prostasin in DU-145 and PC-3 cells reduced the invasiveness by 68% and 42%, respectively, while showing no effect on cell proliferation. The anti-invasion property was associated with cellular prostasin, which exists on cell membrane as an active protease *via* a GPI-anchor. In DU-145 and PC-3 cells expressing recombinant prostasin, a protein band at 120-130 kDa range in SDS-PAGE was found to be reduced in tyrosine phosphorylation while a reduction of protein kinase C alpha expression was also observed. These cellular protein changes elicited by prostasin expression provide leads for investigation of prostasin anti-invasion signal transduction. We demonstrated an up-regulation of hepsin in prostate cancer and are in pursuit of maspin-hepsin interaction by yeast genetics. *Drosophila* genetics will also be employed to investigate hepsin's role in cancer. Our findings may lead to the development of diagnostics or drugs leads.

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FOREWORD

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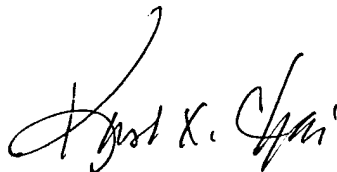
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Table of Contents

        Item 1: Annual Report of the previous year

        Item 2: Paper in press, Chen et al., 2001a

        Item 3: Paper in press, Chen et at., 2001b

(5) INTRODUCTION:

Maspin is a serine protease inhibitor capable of suppressing breast and prostate cancers. The investigation of maspin tumor suppression in breast and prostate cancer cells led to the speculation of maspin's interaction with a membrane-bound serine protease in these tissues or cancers. This interaction may be an initial signaling event to the tumor suppression pathways (manifested as inhibition of tumor cells' motility and invasiveness in *in vitro* and *in vivo* assays, as well as tumor suppression of nude mouse xenografts) (Sheng *et al.*, 1996). We had suggested in our original proposal that a human prostate-produced serine protease, prostasin, can serve the role of a maspin receptor/interactive protease based on our evaluation of prostasin's putative structure and its substrate preference (Yu *et al.*, 1994). We intended to investigate whether a direct interaction between maspin and prostasin can be established using conventional biochemistry and molecular biology methods. We also intended to identify the down-stream proteins in the maspin/prostasin signal transduction pathway taking advantage of *Drosophila* genetics methods. Further, an alternative candidate protease, human hepsin, will also be investigated for the same functions.

(6) BODY:

1). Human prostasin serine protease is a novel tumor invasion suppressor:

Since the most recent annual progress report for the funding period of September 1, 1999 to August 31, 2000, two papers that describe the work funded in this project have been accepted for publication (attached in Appendices).

1. Chen L-M. Hodge GB. Guarda LA. Welch JL. Greenberg NM. Chai KX. Down-regulation of prostasin serine protease, a potential invasion suppressor in prostate cancer. *Prostate*. In press, 2001a

2. Chen L-M. Skinner ML. Kauffman SW. Chao J. Chao L. Thaler CD. Chai KX. Prostasin is a glycosylphosphatidylinositol-anchored active serine protease. *Journal of Biological Chemistry*. In press, 2001b

In the first paper (Chen *et al.*, 2001a), we demonstrated that a prostate-abundant serine protease, prostasin, was absent in highly invasive human prostate cancer cell lines DU-145 and PC-3, while expressed at both the mRNA and the protein levels in normal human prostate epithelial cells and a non-invasive prostate cancer cell line, LNCaP. We further demonstrated that prostasin was down-regulated in high-grade (Gleason 4/5) prostate tumors in prostatectomy specimens. A forced re-expression of recombinant human prostasin protein in DU-145 and PC-3 cells reduced the *in vitro* invasiveness by 68% and 42%, respectively, while showing no effect on cell proliferation. In our invasion assays, we found that it is the cellular prostasin, but not free prostasin purified from human semen or cell culture media, that confers the anti-invasion property

In order to provide structural and regulatory information for further investigation of the functions of prostasin in normal prostate development and prostate cancer, we have investigated the cellular localization of prostasin protein and its biochemical properties inside the cell. The completed work is summarized in the second paper (Chen *et al.*, 2001b). A recombinant human prostasin serine protease (r-hPro) was expressed in several human cell lines, including DU-145 and PC-3. Subcellular fractionation showed that r-hPro is synthesized as a membrane-bound protein, while a free-form prostasin is secreted into the culture medium. Prostasin was identified in nuclear and membrane fractions. Membrane-bound prostasin can be released by phosphatidylinositol-specific phospholipase C treatment, or labeled by [^{3}H]ethanolamine, indicating a glycosylphosphatidylinositol

(GPI)-anchorage. A novel prostasin-binding protein was identified exclusively in seminal vesicle fluid. Both the secreted and the membrane-bound prostasin were able to form a covalently linked 82-kDa complex when incubated with seminal vesicle fluid. The complex formation between prostasin and the prostasin-binding protein was inhibited by a prostasin antibody, heparin, and serine protease inhibitors. Prostasin's serine protease activity was inhibited when bound to the prostasin-binding protein in mouse seminal vesicle fluid. This study indicates that prostasin is an active serine protease in its membrane-bound form. Further, the identification of a specific prostasin-binding protein will facilitate the investigation of prostasin's potential natural substrates or interactive proteins, possibly including maspin, leading the project back to the originally proposed goals of prostasin-maspin interaction.

Because of the new finding that prostasin itself inhibits prostate cancer invasion when re-expressed in DU-145 and PC-3 cells, an investigation of the cellular mechanisms and pathways involved in this process will be highly informative for all potential interactions between prostasin, the serine protease, and its interactive proteins, such as a serine protease inhibitor or an inhibitor-like substrate (e.g., maspin). We have investigated the cellular protein changes resulting from prostasin re-expression in DU-145 and PC-3 cells. DU-145 and PC-3 cells transfected with a vector plasmid or a prostasin expression construct (Chen et al., 2001 a & b) (hereon referred to as DU-145/Vec vs. DU-145/Pro and PC-3/Vec vs. PC-3/Pro) were grown to subconfluency in culture media containing 10% fetal bovine serum (FBS), and harvested by brief trypsinization and neutralization with soybean trypsin inhibitor (SBTI). Cells were washed in OPTI-MEM I serum-free medium (LifeTechnologies, Gaithersburg, MD), rested in OPTI-MEM I for 30 minutes, and seeded at 70-80% confluency with OPTI-MEM I, in 60-mm petri-dishes pre-coated with a thin-layer of ECM gel (used at 3.3 mg/ml, Sigma, St. Louis, MO). After seeding, cells were incubated at 37°C in 5% CO_2 for 60 minutes before being lysed in RIPA buffer (PBS, pH 7.4; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS) containing protease inhibitors and sodium orthovanadate (Sigma, St Louis, MO), a phosphotase inhibitor. Fifty micrograms of total protein for each cell type were analyzed by SDS-PAGE and western blotting using antibodies against human prostasin (Chen et al., 2001 a & b), phosphotyrosine (PY20,), protein kinase C alpha (PKCα), and p120ctn/Cas (BD Transduction Laboratories, San Diego, CA), respectively.

Figure 1
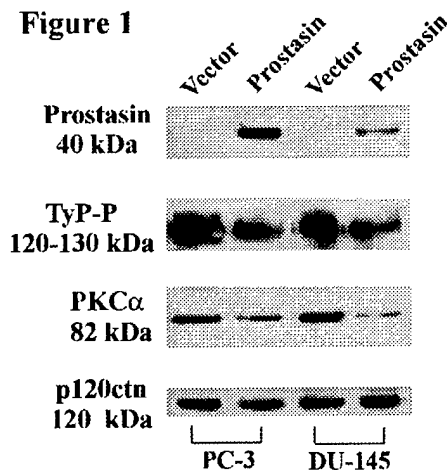
As shown in **Figure 1** (left), DU-145/Pro and PC-3/Pro expressed a 40-kDa prostasin protein while the DU-145/Vec and PC-3/Vec controls did not show prostasin protein expression (top panel). A protein band migrating at approximately 120-130 kDa range in SDS-PAGE was found to be reduced in tyrosine phosphorylation (TyP-P) in DU-145 and PC-3 cells forced to express recombinant human prostasin, as compared to the vector-transfected control cells (second panel from top). Further analysis of the cells demonstrated a consistent reduction of PKCα protein expression in DU-145 or PC-3 cells expressing recombinant prostasin (third panel from top). By employing reciprocal immunoprecipitation/western
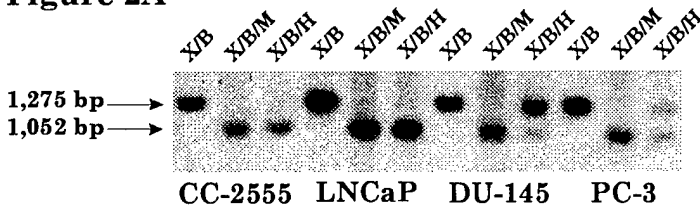
blot analysis, we have ruled out the possibility that the TyP-reduced protein being p120ctn/Cas (data not shown). A p120ctn immunoblot is shown in the bottom panel as protein loading control.

The findings presented in Figure 1 will provide leads for our continued investigation of prostasin's anti-invasion mechanism. For example, the tyrosine phosphorylation-reduced 120-130-kDa protein could be focal adhesion kinase (FAK, 125 kDa) or p130Cas (130 kDa), both proteins have been
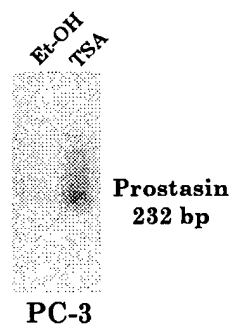
implicated in promoting tumor invasion *via* increased cell motility when tyrosine-phosphorylated (Cary and Guan, 1999). The speculation that either or both of these proteins are reduced in their tyrosine phosphorylation state in the prostate cancer cells expressing recombinant human prostasin is consistent with the fact the prostasin reduced invasiveness. Further, increased PKCα activity has also been implicated in promoting cell motility, and in turn, tumor invasion (Timar *et al.*, 1996). Our observation that PKCα protein expression is reduced as a result of forced prostasin re-expression in the prostate cancer cells is also consistent with prostasin's anti-invasion role.

We investigated the potential molecular mechanisms of prostasin down-regulation in prostate cancer by performing genomic Southern blot analysis of normal prostate epithelial cells, and the LNCaP, DU-145 and PC-3 cell lines. Using a full-length prostasin cDNA probe and 12 different restriction endonucleases, we did not detect any RFLP's (restriction fragment length polymorphisms) or loss of gene at the prostasin locus in these cell lines (data not shown). We amplified a 1,275-bp human prostasin promoter sequence, and used this prostasin promoter DNA as the probe to perform a genomic Southern blot analysis with the methylation-sensitive restriction enzyme *Hpa II*. We have shown that in cells that express prostasin, namely, the normal prostate epithelial cells and the LNCaP, the prostasin promoter is unmethylated, whereas in cells that do not express prostasin, namely the DU-145 and PC-3, the prostasin promoter is heterogeneously hypermethylated (Figure 2A). Treatment of PC-3 cells with the histone acetylase inhibitor trichostatin A (TSA) resulted in re-activation of prostasin mRNA expression (Figure 2B).

Figure 2A

1,275 bp⟶
1,052 bp⟶

CC-2555   LNCaP   DU-145   PC-3

Figure 2B

Prostasin
232 bp

PC-3

Figure 2. Promoter Hypermethylation of the Human Prostasin Gene in Prostate Cancer Cell Lines

A: Genomic DNA (10 μg) from the various cell lines (as indicated in the figure) were digested with the following restriction enzyme combinations, *Xho I/BamH I* (X/B), *Xho I/BamH I/Msp I* (X/B/M), or *Xho I/BamH I/Hpa II* (X/B/H). The digests were resolved in a 0.8% agarose gel and transferred to an Immobilon-N membrane for hybridization with a nick-translated prostasin promoter probe (bases 703 to 1649 of the prostasin gene sequence U33446). The probe detects a promoter fragment of 1,275 *bp* (*Xho I* at base 374 to *BamH I* at base 1649), which is cut by the methylation-insensitive enzyme *Msp I* to yield a 1,052-bp fragment for all DNA samples. The methylation-sensitive isoschizomer *Hpa II* yields the 1,052-bp fragment in the CC-2555 (normal prostate epithelial cells) and the LNCaP samples, indicating the hypomethylated or unmethylated state of the prostasin promoter in these two cells. For the DU-145 and PC-3 cells, however, the *Hpa II* digestion yielded the 1,052-bp and the 1,275-bp fragment, indicating that the *Msp I/Hpa II* site, at location –95 (relative to the transcription initiation site) of the prostasin promoter, is heterogeneously methylated in these two cell lines.

B: Confluent cultures of PC-3 cells in 60-mm dishes were treated for 24 hours with 1 μM trichostatin A (dissolved in 95% ethanol), or with an equal volume of 95% ethanol (as indicated in the Figure). RNA was prepared using the Trizol reagent (LifeTechnologies) and prostasin specific RT-PCR-Southern blot analysis was carried out as described in Chen *et al.*, (2001a).

2).   Maspin, hepsin, and prostasin transgenic flies for the elucidation of the tumor suppression signal pathway(s):

In the previous annual report (appended to this report), we indicated that in the collaborating laboratory (led by Dr. von Kalm), transgenic *Drosophila* strains expressing human maspin, prostasin and hepsin genes were generated and will be applied in *Drosophila* genetic manipulation to identify interactive genes/proteins. The maspin- or prostasin-expressing strains did not exhibit any observable phenotype, consistent with their tumor/invasion suppressor functions or roles in normal prostate tissue physiology. The hepsin-expressing strains all had malformed eyes, which were not corrected upon cross-breeding with the maspin-expressing strains, indicating that the *Drosophila* eye may not provide all necessary cellular pathways for the examination of a potential maspin-hepsin interaction. This observation does not rule out any potential interaction of maspin and hepsin.

We investigated hepsin expression in prostate cancer cell lines and in prostatectomy specimens to support the basis of a potential interaction between maspin and hepsin. In the LNCaP and PC-3 cells, RT-PCR analysis with hepsin-specific primers demonstrated its expression, and *in situ* histochemistry using an anti-sense hepsin RNA probe showed an up-regulation of hepsin in high-grade prostate cancer in all 11 cases of radical prostatectomy examined to date. We obtained a human hepsin antibody from Dr. Kurachi of University of Michigan and attempted to use this antibody in an investigation of direct maspin-hepsin interaction using the human cell lines, as planned in the original Statement of Work. This antibody reagent, however, does not recognize hepsin protein produced in the native form (such as that from the HepG2 cells, the source of the cloned hepsin cDNA), but only reacts with recombinant hepsin produced in *E. coli*. Our attempts of using recombinant hepsin for interaction studies with maspin were hampered by the high degree of difficulty in purifying recombinant hepsin. It is an active serine protease that tends to degrade itself upon purification, but adding protease inhibitors is not applicable in our studies since we need to maintain the serine protease activity of hepsin to evaluate its interaction with maspin. A recent report shows that maspin is capable of inhibiting angiogenesis of human prostate cancer cells injected into animals (Zhang *et al.*, 2000). Given the involvement of hepsin in angiogenesis (activating Factor VII) and its up-regulation in prostate cancer (Kazama *et al.*, 1995), we consider the likelihood of an interaction between maspin and hepsin being very high. Due to our lack of reactive hepsin antibody, we solicited the assistance of Dr. Ming Zhang of Baylor College of Medicine to perform a yeast two-hybrid system assay on maspin and hepsin. The work is currently underway in Dr. Zhang's laboratory. The transgenic *Drosophila* strains that express hepsin in the eyes will be used to identify other potential interactive proteins for hepsin, a tumor-promoting serine protease (Torres-Rosado *et al.*, 1993) and a potential prostate cancer marker.

Based on our most recent data presented in Figure 1, prostasin's anti-invasion mechanism may involve regulation of cell motility, which implicates the importance of cytoskeleton. In *Drosophila*, the stubble, a potentially membrane-bound serine protease, has been shown by genetic means to affect cytoskeleton organization, in that fly legs are malformed in stubble mutants (von Kalm *et al.*, 1995). Dr. von Kalm's laboratory is among the leaders in the study of stubble serine protease's cellular signaling pathways and has the full complement of all stubble serine protease genetic strains. Work is now currently underway to create prostasin transgenic flies to cross with stubble mutants with the purpose of addressing a very specific question: will prostasin rescue the malformation of fly legs in the stubble mutants.

(7) KEY RESEARCH ACCOMPLISHMENTS:

_____ Prostasin expression is found to be down-regulated in high-grade prostate cancer. Analysis of the prostasin promoter region in prostate cancer cell lines indicated DNA hypermethylation as a potential epigenetic mechanism of prostasin gene-silencing in prostate cancer.

_____ Prostasin was shown to be a potential invasion suppressor of prostate cancer.

_____ Prostasin's sub-cellular localization has been thoroughly investigated and prostasin's membrane anchorage was confirmed.

_____ A novel prostasin-binding protein has been identified in mouse seminal vesicle. Using this serpin-like protein (in the context of the seminal vesicle extract mixture), we were able to show that the membrane-bound prostasin is an active serine protease. The confirmation of membrane-prostasin serine protease activity will also provide important leads in the investigation of the mechanisms of prostasin's biological functions.

_____ Prostasin re-expression in human prostate cancer cell lines DU-145 and PC-3 elicited cellular protein changes that potentially could implicate prostasin in regulating cell motility. These cellular protein changes are the reduction of tyrosine phosphorylation of a 120-130-kDa protein, suspected to be FAK or p130Cas; and the down-regulation of PKCα protein expression.

_____ The speculated role of prostasin in regulating cell motility led to the undertaking of transgenic fly work to investigate whether prostasin is capable of rescuing stubble serine protease mutants that display leg malformation.

(8) REPORTABLE OUTCOMES:

– manuscripts, abstracts, presentations;

The following two papers are in press:

1. Chen L-M. Hodge GB. Guarda LA. Welch JL. Greenberg NM. Chai KX. Down-regulation of prostasin serine protease, a potential invasion suppressor in prostate cancer. _**Prostate**_.

2. Chen L-M. Skinner ML. Kauffman SW. Chao J. Chao L. Thaler CD. Chai KX. Prostasin is a glycosylphosphatidylinositol-anchored active serine protease. _**Journal of Biological Chemistry**_.

The following presentation was made at the meeting indicated:
Chen, L-M., Suffoletto, M., Thaler, C. D., Hodge, G. B., Guarda, L. A., Welch, J. L., Greenberg, N. M., Chao, L., Chao, J., and Chai, K. X. (2000) Prostasin Serine Protease in Prostate Cancer and Identification of A Prostasin-binding Protein. The Society for Basic Urologic Research Fall Symposium at Sanibel Harbour Resort and Spa, Florida.

– patents and licenses applied for and/or issued;

A full utility patent application under the title of "A Method of Identifying and Treating Invasive Carcinomas" has been filed with the USPTO. Co-inventors: Karl X. Chai, Li-Mei Chen, Lee Chao, and Julie Chao.

– degrees obtained that are supported by this award;

Shabnam Dabirshahsahebi, Master of Science, 2000. Thesis "Genetic Analysis of Human Serine proteases and Serpins by Expression in the _Drosophila_ Eye", University of Central Florida

− funding applied for based on work supported by this award;

1. DoD PCRP01, "Prostasin's Role in Prostate Cancer" Principal Investigator: Karl X. Chai, Ph.D. Type: Idea Award, total direct cost applied for: $361,123

2. National Institutes of Health, R03 HD40241-01 "The Role of Serine Protease Inhibitors in Fertility", Principal Investigator: Li-Mei Chen, M.D., Ph.D. Total direct cost applied for: $100,000 (re-submitted on 03/01/01)

(9) CONCLUSIONS:

We have found that human prostasin is a suppressor of prostate cancer cell chemoinvasiveness and have discovered a molecular mechanism for its down-regulation in prostate cancer. We have investigated prostasin's cellular and biochemical properties and paved the way for an in-depth investigation of the mechanisms of prostasin's biological functions.

"So What Section" The currently completed research has the following implications toward the diagnosis or treatment of human prostate cancer:

1). An assay (test) may be established to determine the level of production of human prostasin in prostate cancer using an immunological reagent (antibody) for diagnosis or prognosis of the invasiveness of prostate cancer since we have demonstrated a correlation between prostasin's down-regulation and prostate cancer grade.

2). A molecular genetic assay (test) may also be established to determine the methylation state of the prostasin promoter in prostate cancer and the result may be applicable to prostate cancer diagnosis or prognosis.

3). Our demonstration of prostasin serine protease activity in the membrane-bound form may lead us to the identification of prostate cancer relevant molecules. The identification and investigation of these molecules will offer us better understanding of the progression of prostate cancer and may lead to the development of diagnostics and drugs.

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·13. ABSTRACT *(Maximum 200 Words)*

The purpose of this project was to identify a maspin receptor, and to investigate the signal transduction in maspin-induced cancer suppression. Human prostasin and hepsin were examined as candidate maspin receptor serine proteases. We had shown a down-regulation of prostasin in prostate cancer, correlating with cancer grade, eliminating this protease as a maspin receptor. We confirmed prostasin's anti-invasion function in polyclonal PC-3 cells transfected with prostasin cDNA. We are investigating prostasin's *in vivo* anti-invasion function in a nude mouse model *via* intravenous or orthotopic tumor injection. DNA hypermethylation was discovered in the prostasin gene promoter in cancer cells that do not express prostasin. We confirmed that prostasin protein is membrane-anchored *via* a GPI-linakge, determined its sub-cellular localization in the ER/Golgi/nuclear complex and the nucleus, and confirmed the serine protease activity for the membrane-bound prostasin by using a novel prostasin-binding protein. The prostasin-binding protein was discovered in mouse seminal vesicle and was found to be a serpin-like protein. We demonstrated an up-regulation of hepsin in prostate cancer and are in pursuit of maspin-hepsin interaction by yeast genetics. *Drosophila* genetics will be employed to investigate hepsin's role in cancer. Our findings may lead to the development of diagnostics or drugs leads.

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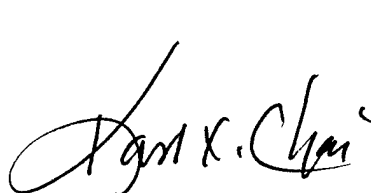
X_ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X_ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.
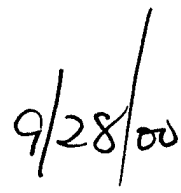
X_ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

X_ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

X_ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

_____   9/28/00
PI - Signature   Date

(4) TABLE OF CONTENTS:

(5) INTRODUCTION:

Maspin is a serine protease inhibitor that was found to suppress breast and prostate cancers. The investigation of this tumor suppression in breast and prostate cancer cells led to the speculation of maspin's interaction with a membrane-bound serine protease in these tissues or cancers. This interaction may be an initial signaling event to the tumor suppression pathways (manifested as inhibition of tumor cells' motility and invasiveness in *in vitro* and *in vivo* assays) (Sheng *et al.*, 1996). We had suggested in our original proposal that a human prostate-produced serine protease, prostasin, can serve the role of a maspin receptor/interactive protease based on our evaluation of prostasin's putative structure and its substrate preference (Yu *et al.*, 1994). We intended to investigate whether a direct interaction between maspin and prostasin can be established using conventional biochemistry and molecular biology methods. We also intended to identify the down-stream proteins in the maspin/prostasin signal transduction pathway taking advantage of *Drosophila* genetics methods. Further, an alternative candidate protease, human hepsin, will also be investigated for the same functions.

(6) BODY:

1). Human prostasin serine protease is a novel tumor invasion suppressor:

In our first annual progress report for the funding period of September 1, 1998 to August 31, 1999, we had shown that human prostasin is down-regulated in prostate cancer and prostasin re-expression in PC-3 cells reduces invasiveness. We now have expanded our data to show a definitive prostasin down-regulation in prostate cancer.

Our cell line evaluation now had included a normal prostate epithelial cell, the LNCaP, DU-145, and PC-3 human prostate cancer cell lines, and three prostate cancer cell lines from the TRAMP animal model (Figure 1). In all cell lines evaluated, only the LNCaP cells, which is the least invasive, showed prostasin expression along with the normal prostate epithelial cells.

Our prostatectomy *in situ* histochemistry studies now had collected data from 35 patients and we show a correlation between prostate cancer grade and prostasin down-regulation (Figure 2). In all specimens that were analyzed, prostasin was detected in benign areas (+++), low Gleason grade areas ($\leq$ grade 2: ++; grade 3: $\pm$); but not in high Gleason grade areas ($\geq$ grade 4: -).

In an *in vitro* Matrigel invasion assay, we evaluated the invasiveness and maspin expression of the prostasin-expressing PC-3 cells that were clonally selected. As a result, we have shown that maspin's expression is induced by the introduction of prostasin in PC-3 cells while the *in vitro* chemoinvasiveness of the cells is reduced. We have repeated our experiments with an episomal expression system (Invitrogen, Madison, WI) to establish polyclonal PC-3 cells that express prostasin or carry the control vector. We were able to confirm the reduction of PC-3 chemoinvasiveness (Figure 3), but were not able to show maspin expression difference. The maspin expression difference that we observed previously is attributed to clonal selection.

We then investigated the potential effects of prostasin expression in PC-3 cells on cell growth. In an *in vitro* MTT metabolic cell growth assay, we cultured PC-3 cells, PC-3 carrying the control vector and two independent polyclonal transfectants of PC-3 that express human prostasin. Over a period of 8 days, the cells did not show any difference in growth rate (data not shown). With funding provided by Florida Hospital and an IACUC approval, we investigated the tumor take and growth rate of transgrafted tumors by injecting these cells subcutaneously into male nude mice. Over a 6-

week observation period, there was no difference in tumor take or growth between these cells (data not shown). At this point, we are hypothesizing that prostasin is a normal prostate epithelial cell protein that is down-regulation in high-grade cancer, its biological function may be inhibition of cell invasion and its down-regulation may be a prerequisite for tumor invasion and metastasis. We are now at week-4 of a 6-week observation period in an evaluation of prostasin's anti-invasion/anti-metastasis function using a tail-vein tumor inoculation model. These various PC-3 cells were injected into the tail vein of male nude mice (700,000 cells per animal) and we will examine the incidence of lung colonization following a 6-week observation period (ending in mid-October). In a separate project, orthotopic tumor injection of these various PC-3 cells into the prostate of male nude mice will be performed to evaluate the potential growth differences influenced by the proper micro-tissue environment (as opposed to the subcutaneous sites).

We investigated the potential molecular mechanisms of prostasin down-regulation in prostate cancer by performing genomic Southern blot analysis of normal prostate epithelial cells, and the LNCaP, DU-145 and PC-3 cell lines. Using a full-length prostasin cDNA probe and 12 different restriction endonucleases, we did not detect any RFLP's (restriction fragment length polymorphisms) or loss of gene at the prostasin locus in these cell lines (data not shown). We amplified a 1,275-bp human prostasin promoter sequence, and used this prostasin promoter DNA as the probe to perform a genomic Southern blot analysis with the methylation-sensitive restriction enzyme *Hpa II*. We have shown that in cells that express prostasin, namely, the normal prostate epithelial cells and the LNCaP, the prostasin promoter is unmethylated, whereas in cells that do not express prostasin, namely the DU-145 and PC-3, the prostasin promoter is hypermethylated (Figure 4).

We have begun our quest in investigating the potential biochemical or cellular mechanisms of prostasin's anti-invasive function. Our first finding was the confirmation of prostasin's membrane anchorage. Prostasin protein was localized in various cellular sub-fractionations (Figure 5) and it can be either extracted with the detergent Triton X-114 or released upon phospholipase C treatment (data not shown), indicating a GPI-anchorage. By confocal microscopy, we confirmed the localization of prostasin in the ER/Golgi/nuclear complex (Figure 6), providing leads to our future investigation of prostasin's cellular function. Our second major finding was the confirmation of prostasin's serine protease activity in the membrane-bound form. We identified a novel prostasin-binding protein in mouse seminal vesicle (Figure 7) by performing a serine protease-binding assay as described previously (Chen *et al.*, 1990). The new prostasin-binding protein provided a means of investigating this serine protease's activity in the membrane-bound form (in a mixture with other membrane-bound proteins, a situation in which synthetic substrates are not applicable). The membrane-bound prostasin can form a covalent complex with the new prostasin-binding protein (Figure 8), and the complex is inhibited by pre-incubation with aprotinin, indicating that the interaction in through the active site serine residue. These findings of prostasin's biochemical and cellular properties will guide us in the future studies of prostasin's anti-invasion mechanisms, a goal originally designed and approved for the study of maspin action, but adapted for prostasin, a new tumor invasion suppressor.

2). Maspin, hepsin, and prostasin transgenic flies for the elucidation of the tumor suppression signal pathway(s):

Upon a thorough interrogation of the *Drosophila* genome database, we had found a high number of candidate genes for maspin, prostasin or hepsin homologues in flies. All of these candidate genes share an extensive sequence homology with the human target genes and without any information regarding the function or even the expression pattern of these potential candidate genes,

identification of the true homologues of human genes was deemed impossible. In the collaborating laboratory (led by Dr. von Kalm), transgenic *Drosophila* strains expressing human maspin, prostasin and hepsin genes were generated and will be applied in *Drosophila* genetic manipulation to identify interactive genes/proteins. The maspin- or prostasin-expressing strains did not exhibit any observable phenotype, consistent with their tumor suppressor functions or roles in normal prostate tissue physiology. The hepsin-expressing strains all had malformed eyes (Figure 9), which were not corrected upon cross-breeding with the maspin-expressing strains (data not shown), indicating that the *Drosophila* eye may not provide all necessary cellular pathways for the examination of a potential maspin-hepsin interaction. This observation does not rule out any potential interaction of maspin and hepsin.

We investigated hepsin expression in prostate cancer cell lines and in prostatectomy specimens to support the basis of a potential interaction between maspin and hepsin. In the LNCaP and PC-3 cells, RT-PCR analysis with hepsin-specific primers demonstrated its expression (data not shown), and *in situ* histochemistry using an anti-sense hepsin RNA probe showed an up-regulation of hepsin in high-grade prostate cancer in all 11 cases of radical prostatectomy examined to date (Figure 10). We obtained a human hepsin antibody from Dr. Kurachi of University of Michigan and attempted to use this antibody in an investigation of direct maspin-hepsin interaction using the human cell lines, as planned in the original Statement of Work. This antibody reagent, however, does not recognize hepsin protein produced in the native form (such as that from the HepG2 cells, the source of the cloned hepsin cDNA), but only reacts with recombinant hepsin produced in *E. coli*. Our attempts of using recombinant hepsin for interaction studies with maspin were hampered by the high degree of difficulty in purifying recombinant hepsin. It is an active serine protease that tends to degrade itself upon purification, but adding protease inhibitors is not applicable in our studies since we need to maintain the serine protease activity of hepsin to evaluate its interaction with maspin. A recent report shows that maspin is capable of inhibiting angiogenesis of human prostate cancer cells injected into animals (Zhang *et al.*, 2000). Given the involvement of hepsin in angiogenesis (activating Factor VII) and its up-regulation in prostate cancer (Kazama *et al.*, 1995), we consider the likelihood of an interaction between maspin and hepsin being very high. Due to our lack of reactive hepsin antibody, we solicited the assistance of Dr. Ming Zhang of Baylor College of Medicine to perform a yeast two-hybrid system assay on maspin and hepsin. The work is currently underway in Dr. Zhang's laboratory. The transgenic *Drosophila* strains that express hepsin in the eyes will be used to identify other potential interactive proteins for hepsin, a tumor-promoting serine protease (Torres-Rosado *et al.*, 1993) and a potential prostate cancer marker.

(7) KEY RESEARCH ACCOMPLISHMENTS:

_____ Prostasin expression is found to be down-regulated in prostate cancer and the down-regulation is correlated to cancer grade. Analysis of the prostasin promoter region in four prostate cancer or normal cell lines indicated DNA hypermethylation as a potential epigenetic mechanism of prostasin gene silencing in prostate cancer.

_____ Human prostate cancer bone metastasis cell line PC-3 transfected with a full-length human prostasin cDNA displayed a significantly reduced level of invasiveness (42% inhibition) in an *in vitro* Matrigel invasion assay for polyclonal transfectants (as opposed to the clonally selected transfectants reported in the previous year).

_____ Prostasin's sub-cellular localization has been thoroughly investigated and the information guides our future research of the mechanisms of prostasin's biological functions. Prostasin's membrane anchorage was confirmed in these studies.

_____ A novel prostasin-binding protein has been identified in mouse seminal vesicle. Using this serpin-like protein (in the context of the seminal vesicle extract mixture), we were able to show that the membrane-bound prostasin is an active serine protease. The confirmation of membrane-prostasin serine protease activity will also provide important leads in the investigation of the mechanisms of prostasin's biological functions.

_____ Human hepsin mRNA was found over-expressed in prostate cancer, providing the basis of a potential interaction between maspin and this second candidate receptor serine protease.

(8) REPORTABLE OUTCOMES:

- manuscripts, abstracts, presentations;

The following two manuscripts are in revision for re-submission:

1. Chen L-M. Suffoletto M. Hodge GB. Guarda LA. Welch JL. Greenberg NM. Chai KX. Prostasin Serine Protease Is Down-regulated in Prostate Cancer, and Reduces Chemoinvasion of PC-3 Cells upon Transfection. Revision.
2. Chen L-M. Skinner ML. Kauffman SW. Chao J. Chao L. Thaler CD. Chai KX. Expression of GPI-Anchored Recombinant Human Prostasin and Identification of A Novel Prostasin-binding Protein. Revision (will be re-submitted to Journal of Biological Chemistry)

The following presentations have been or will be made at the meetings listed:
1. Dabirshahsahebi, S., Willey, J., Bayer, C., Leppert, A., Chai, K. X., and von Kalm, L. (1999) Genetic Analysis of Vertebrate Serine Proteases and Serpins by Mis-expression in the *Drosophila* Eye. Presented at the Southeast Regional Drosophila Meeting, Emory University, Atlanta, GA.

2. Chen, L-M., Hodge, G. B., Guarda, L. A., Welch, J. L., Greenberg, N. M., Chao, L., Chao, J., and Chai, K. X. (2000) Prostasin Serine Protease in Prostate Cancer and Identification of A Prostasin-binding Protein. Will be presented at the Society for Basic Urologic Research Fall Symposium at Sanibel Harbour Resort and Spa, Florida.

- patents and licenses applied for and/or issued;

A provisional patent application under the title of "A Method of Identifying and Treating Invasive Carcinomas" has been filed with the USPTO. Co-inventors: Karl X. Chai, Li-Mei Chen, Lee Chao, and Julie Chao.

- degrees obtained that are supported by this award;

James A. Murphy, Master of Science, 2000. Thesis "The Serine Proteinase Inhibitor, Maspin Does Not Interact with Prostasin in Controlling the Invasive Phenotype of Breast and Prostate Cancer Cells", University of Central Florida

- funding applied for based on work supported by this award;

1. DoD PCRP00, "Prostasin Serine Protease as A Prostate Cancer Marker" Principal Investigator: Karl X. Chai, Ph.D. Type: Idea Award, total direct cost applied for: $117,436

2. DoD PCRP00, "Hepsin's Role in Prostate Cancer" Principal Investigator: Laurence von Kalm, Ph.D. Co-investigator: Karl X. Chai, Ph.D. Type: New Investigator Award, total direct cost applied for: $225,000

3. National Institutes of Health, R03 HD40241-01 "The Role of Serine Protease Inhibitors in Fertility", Principal Investigator: Li-Mei Chen, M.D., Ph.D. Total direct cost applied for: $100,000

(9) CONCLUSIONS:

We have found that human prostasin is a suppressor of prostate cancer cell chemoinvasiveness and have discovered a molecular mechanism for its down-regulation in prostate cancer. We have investigated prostasin's cellular and biochemical properties and paved the way for an in-depth investigation of the mechanisms of prostasin's biological functions. We have found that the hepsin serine protease is up-regulated in prostate cancer at the mRNA level.

"So What Section" The currently completed research has the following implications toward the diagnosis or treatment of human prostate cancer:

1). An assay (test) may be established to determine the level of production of human prostasin in prostate cancer using an immunological reagent (antibody) for diagnosis or prognosis of the invasiveness of prostate cancer since we have demonstrated a correlation between prostasin's down-regulation and prostate cancer grade.

2). A molecular genetic assay (test) may also be established to determine the methylation state of the prostasin promoter in prostate cancer and the result may be applicable to prostate cancer diagnosis or prognosis.

3). Our demonstration of prostasin serine protease activity in the membrane-bound form may lead us to the identification of prostate cancer relevant molecules. The identification and investigation of these molecules will offer us better understanding of the progression of prostate cancer and may lead to the development of diagnostics and drugs.

4). Hepsin mRNA up-regulation in prostate cancer may be pursued as another prostate cancer marker or target for drugs.

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Zhang M. Volpert O. Shi YH. Bouck N. Maspin is an angiogenesis inhibitor. Nature Medicine. 6(2):196-9, 2000

12

(11) APPENDICES:

Appendix 1. Figures and Tables
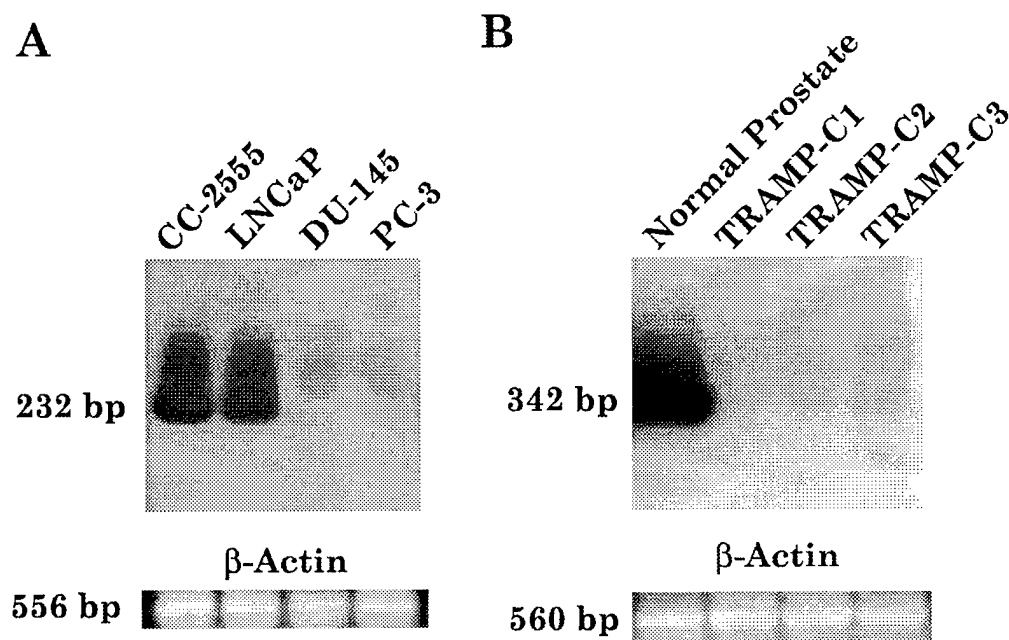
Figure 1.   RT-PCR-Southern Blot Analysis of Prostasin Expression in Prostate Cells or Prostate Cancer Cell Lines

Panel A: RT-PCR-Southern blot analysis using human prostasin-specific oligonucleotides. The samples loaded in each lane are as indicated in the figure. Prostasin mRNA (*via* a 232-bp amplified DNA band) is detected in normal prostate epithelial cells (CC-2555) and the LNCaP cells, but not the DU-145 or PC-3 cells. Co-amplification of a 556-bp human β–actin message (as shown in the gel photograph in the lower panel) confirmed the quality and the quantity of the RNA applied in each RT-PCR.

Panel B: Total RNA (1 µg) from a normal mouse prostate (whole), and the TRAMP-C cells were subjected to an RT-PCR using two mouse prostasin-specific oligonucleotide primers. The amplified mouse prostasin message (a cDNA band of 342 bp) was then probed with a full-length human prostasin cDNA, as shown in the autoradiogram in the upper panel. The samples loaded in each lane are as indicated in the figure. Note the strong signal of mouse prostasin message in the normal prostate RNA sample, in contrast to the 342-bp band-negative lanes of TRAMP-C1, C2 and C3. Co-amplification of a 560-bp mouse β–actin message (as shown in the gel photograph in the lower panel) confirmed the quality and the quantity of the RNA applied in each RT-PCR.
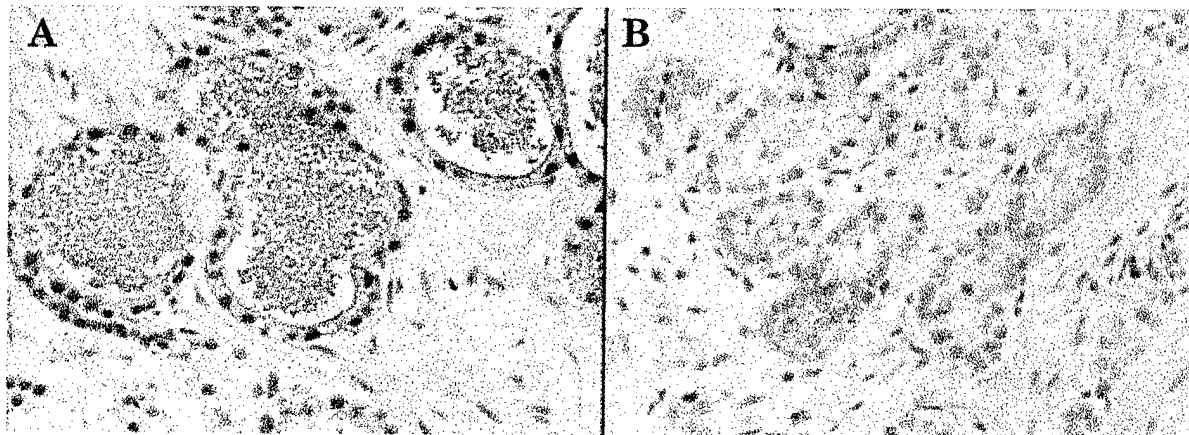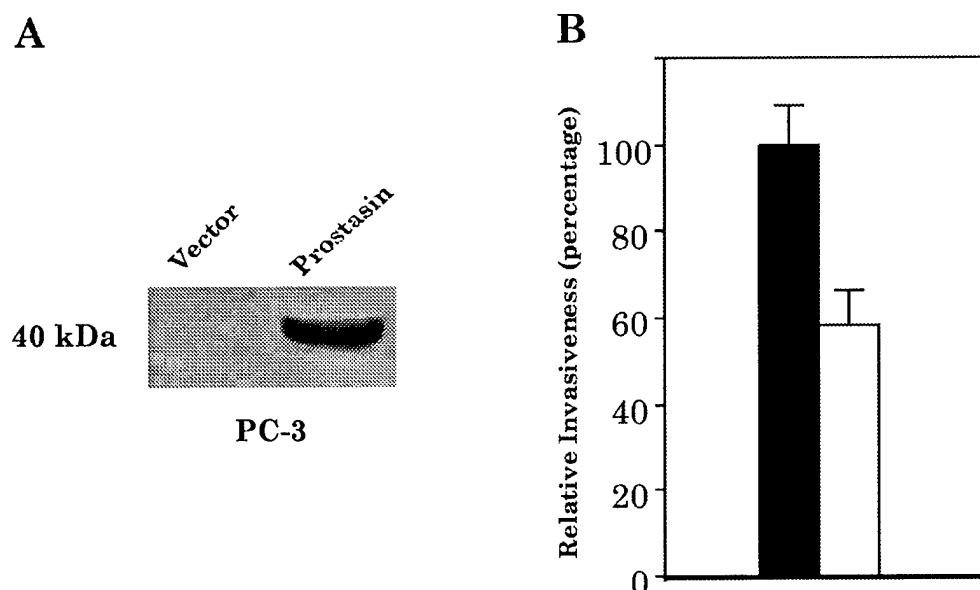
Figure 2.   Prostasin Immunostaining in Human Prostate

Paraffin-embedded human prostatectomy sections were stained for prostasin protein using a specific antibody.   Panel A: a non-cancerous, or benign area of a section of the prostate showing prostasin-specific staining (brown color) in the epithelial cells as well as in the secretion in the lumen (image magnified at 200 x).

Panel B: a tumor area of the same section showing none, or very little prostasin-specific staining (only the blue counter-stain is present). The images presented here are from a prostate tumor with a Gleason score of 3+3 (image magnified at 200 x).

A   **B**

Figure shows Western blot (40 kDa, Vector/Prostasin lanes, PC-3) and bar graph of Relative Invasiveness (percentage).

Figure 3A. Expression of Human Prostasin Protein in Transfected PC-3 Cells

Total protein extracts (50 µg/sample) were subjected to an SDS-PAGE followed by a western blot analysis using a prostasin-specific antibody. The lane labeled "Vector" represents lysate from the cells transfected with the vector plasmid, the lane labeled "Prostasin" represents lysate from the cells transfected with a full-length human prostasin cDNA construct. The expressed human prostasin protein (a 40-kDa band) is detected in the prostasin cDNA-transfected PC-3 cells, but not in the vector-alone transfected cells.

3B.   *In Vitro* Matrigel Invasion Assays of the PC-3 Transfectants

PC-3 cells transfected with either the vector DNA alone or the prostasin cDNA construct, were subjected to an *in vitro* Matrigel invasion assay. The data represent three invasion assays, and each assay was performed using triplicate data points (filters) for each cell type. The vector-alone transfected cells are expressed as being 100% invasive (solid bar), the open bar represents the relative invasiveness of the cancer cells expressing human prostasin. The data were analyzed by a Student *t*-test using the StatView software (Abacus Concepts, Inc., Berkeley, CA) ($p < 0.01$).
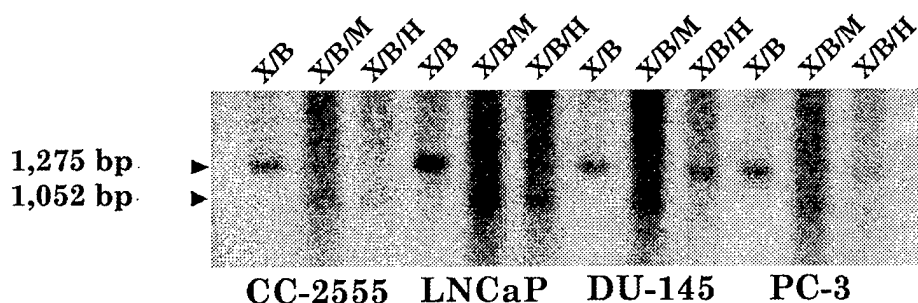
Figure 4. Promoter Hypermethylation of the Human Prostasin Gene

Genomic DNA (10 µg) from the various cell lines (as indicated in the figure) were digested with the following restriction enzyme combinations, *Xho I*/*BamH I* (X/B), *Xho I*/*BamH I*/*Msp I* (X/B/M), or *Xho I*/*BamH I*/*Hpa II* (X/B/H). The digests were resolved in a 0.8% agarose gel and transferred to an Immobilon-N membrane for hybridization with a nick-translated prostasin promoter probe (bases 703 to 1649 of the prostasin gene sequence U33446). The probe detects a promoter fragment of 1,275 bp (*Xho I* at base 374 to *BamH I* at base 1649), which is cut by the methylation-insensitive enzyme *Msp I* to yield a 1,052-bp fragment for all DNA samples. The methylation-sensitive isoschizomer *Hpa II* yields the 1,052-bp fragment in the CC-2555 (normal prostate epithelial cells) and the LNCaP samples, indicating the hypomethylated or unmethylated state of the prostasin promoter in these two cells. For the DU-145 and PC-3 cells, however, the *Hpa II* digestion did not yield the 1,052-bp but the 1,275-bp fragment, indicating that the *Msp I*/*Hpa II* site, at location −95 (relative to the transcription initiation site) of the prostasin promoter, is methylated (hypermethylated) in these two cells.

40kDa

Pro P1   P2   P3   S
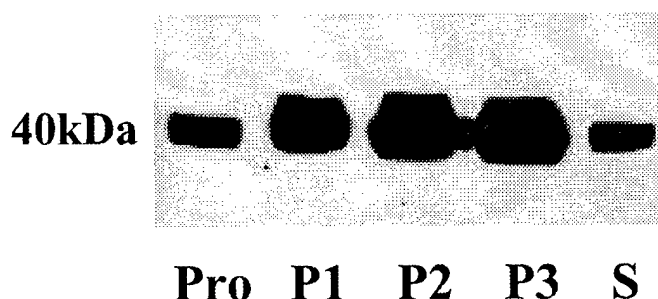
Figure 5. Differential Centrifugation and Western Blot Analysis of Cellular Prostasin Protein
The prostasin protein is found at the nucleus (P1, 500-g pellet), heavy membranes (P2, 10,000-g
pellet, containing mitochondria, lysosomes, and peroxisomes), light membranes (P3, 150,000-g
pellet, containing plasma membrane, microsomes, and the endoplasmic reticulum), and the cytosol
(S, supernatant at 150,000 x g). These data support the possibility that the prostasin protein can
assume a membrane-bound form, which is distributed throughout the cellular compartments
validating our immunohistochemistry results on human prostate specimens. At the same time, we
also detected the prostasin protein in the culture media of the 293-Pro cells, indicating that part of the
prostasin protein synthesized is secreted outside the cell.

Figure 6. Confocal Microscopy Localization of Prostasin in PC-3 Cells Tranfected with A Prostasin Plasmid

Cells were immuno-stained with prostasin polyclonal antibody (seen in green) and transportin monoclonal antibody (seen in red). Prostasin is detected on the plasma membrane (blue arrow), cytoplasma (bright yellow arrow), and the nuclear/ER/Golgi network as well as the nucleus (magenta arrow). Transportin is a nucleoprotein detected in the nucleus. Both proteins co-localize at the nuclear/RE/goli network and nucleus (psuedo-yellow superimposed image). Magnification: 400X.
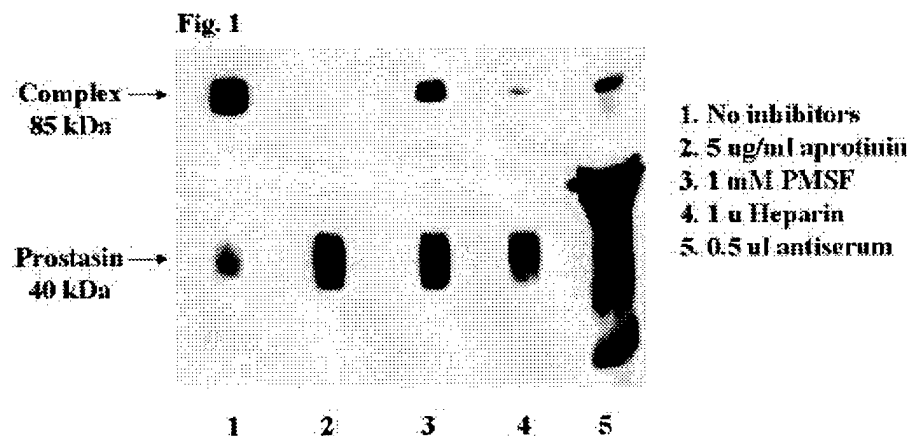
Figure 7. Identification of A Novel Prostasin-binding Protein in Mouse Seminal Vesicle

Lane 1, purified human prostasin was incubated with mouse seminal vesicle fluid; lanes 2-5, prostasin was pre-incubated with serine protease inhibitors, heparin, or a prostasin-specific antibody for 15 min at room temperature before an incubation with mouse seminal vesicle fluid for another 60 min at 37°C. The complex formation was abolished with a pre-incubation of aprotinin (5 μg/ml), or was significantly reduced with a pre-incubation of PSMF (1 mM), heparin (1 unit), or the prostasin antiserum (0.5 μl). The proteins were resolved by SDS-PAGE and were detected by western blot analysis using a polyclonal prostasin antibody.

82 kDa

40 kDa

P1 P2 P3
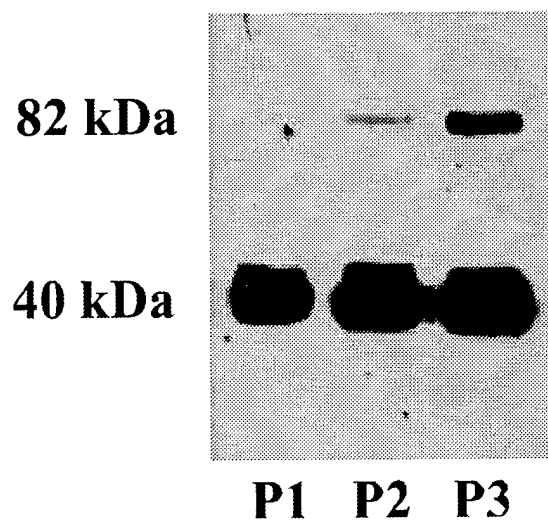
Figure 8. Complex Formation between Membrane-bound Prostasin and A Putative New Serpin in Mouse Seminal Vesicle

Mouse seminal vesicle extract (10 µl) was incubated with sub-cellular fractions (extracted in the absence of serine protease inhibitors) at 37°C for 60 min. The binding reaction is stopped by the addition of SDS-sample buffer and the mixture is heated at 100°C for 5 min. An SDS-PAGE followed by western blot analysis with a prostasin-specific antibody, was used to detect the prostasin-serpin complex at 82 kDa.
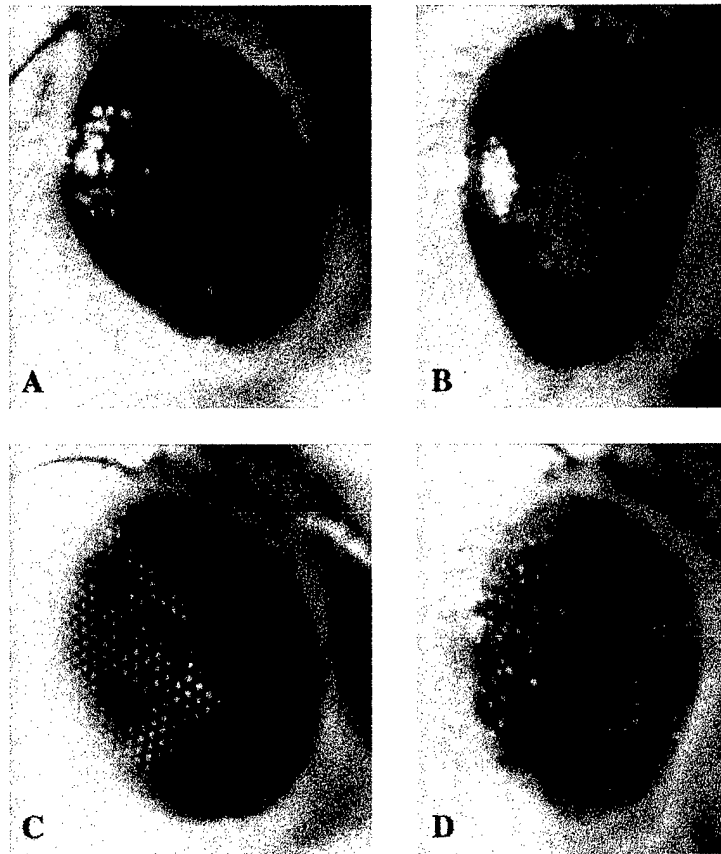
Figure 9: Expression of human hepsin in the Drosophila eye generates a mutant eye phenotype. **A:** Expression of 1 copy of the hepsin transgene under the control of the eye-specific GMR enhancer. The GMR enhancer causes the hepsin transgene to be activated in a glass-like expression domain. The ommatidial array is disorganized resulting in an easily scored rough eye phenotype that is invariant across the eye. **B:** Expression of 2 copies of the hepsin transgene under the control of the GMR enhancer. The eye has a glassy appearance with no evidence of an ommatidial array and its center is often unpigmented. **C:** A wild-type (normal) eye. The ommatidial array is highly organized. **D:** Expression of a hepsin transgene driven by the eye-specific sevenless enhancer generates a rough eye phenotype with variable morphology within the eye. The sevenless enahncer causes hepsin to be expressed in a subset of photoreceptor neurons.

Figure 10. *In Situ* **Localization of Hepsin mRNA in Human Prostate Cancer**

A human hepsin anti-sense RNA probe was prepared and used to hybridize with human hepsin mRNA in a section (0.5 μM) of a human prostate removed by radical prostatectomy. Panel A shows hepsin mRNA signal in the prostate epithelial cells in a Gleason grade-3 tumor area, while Panel B shows a benign area from the same section, which has no hepsin mRNA signal.

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Down-regulation of Prostasin Serine Protease, A Potential Invasion Suppressor in Prostate Cancer

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Running Title: Prostasin Down-regulation in Prostate Cancer

ABSTRACT

BACKGROUND. Prostasin is a serine protease predominantly expressed in normal prostate epithelial cells. The biological function of prostasin has not been determined.

METHODS. Western blot and RT-PCR analyses were used to examine the expression of prostasin in prostate cancer cell lines. Immunohistochemistry was used to evaluate prostasin protein expression in human prostate cancer. An *in vitro* Matrigel invasion assay was used to test the invasiveness of prostate cancer cell lines forced to express recombinant prostasin.

RESULTS. Both prostasin protein and mRNA were found to be expressed in normal human prostate epithelial cells and a non-invasive human prostate cancer cell line, the LNCaP, but neither was found in invasive human prostate cancer cell lines DU-145 and PC-3. Prostasin mRNA expression was absent in invasive prostate cancer cell lines of a transgenic mouse model. Immunohistochemistry analysis showed that prostasin protein expression is down-regulated in high-grade prostate cancer. Transfection of DU-145 and PC-3 cells with a full-length human prostasin cDNA restored prostasin expression and reduced the *in vitro* invasiveness by 68% and 42%, respectively.

CONCLUSIONS. Our data indicate that prostasin may be implicated in normal prostate biology and is able to suppress prostate cancer invasion *in vitro*.

KEY WORDS: cell line; gene expression; immunohistochemistry; prostatectomy; transfection

INTRODUCTION

For men in the U.S., prostate cancer is the most commonly diagnosed cancer, and the second leading cause of cancer-related death [1]. The primary goals of prostate cancer research are to find new markers or assays for early detection, and new methods to control invasion and metastasis. Our laboratory's research focus is on the roles played by serine proteases and serine protease inhibitors in the development or progression of cancer. In this report we describe the results on a serine protease, prostasin [2].

The conventional paradigm of protease involvement in the development and progression of cancer has been the assignment of a usually negative role to the proteases, such as promoting tumor invasion [3]. In turn, the conventional paradigm of protease inhibitors in relation to cancer is usually a regard of a beneficial effect for the presence of these molecules [4]. Recently, however, the picture of a new paradigm is beginning to emerge for several serine proteases in breast, prostate, and testicular cancers. A "normal epithelial cell specific-1" (NES1) serine protease was found to be down-regulated in breast and prostate cancers, and it functions as a tumor suppressor [5]. A prostate-specific serine protease, prostase [6], was shown to be expressed in normal prostate but not in prostate cancer cell lines DU-145 and PC-3. The expression of a testis-specific serine protease, testisin, was shown to be lost in testicular cancer through either a loss of gene [7] or methylation in the promoter [8]. Further, transfection of human testicular cancer cells with a testisin cDNA reduced the tumor growth of xenografts of these cells in nude mice, suggesting a tumor suppressor function for testisin [8]. The testisin serine protease is potentially membrane-bound as suggested by its structure and confirmed by immunohistochemistry [7].

Prostasin serine protease is an acidic protein (pI 4.5-4.8) of approximately 40 kDa in molecular mass [2]. It is predominantly made in the prostate gland (~140 ng/mg protein), with lesser amounts (2-6 ng/mg protein) also found in the bronchi, colon, kidney, liver, lung,

pancreas, and the salivary glands [2]. Prostasin is secreted in the prostatic fluid, and can be detected in the semen (~9 μg/ml). Prostasin expression is localized to the epithelial cells of human prostate gland by *in situ* hybridization histochemistry using an antibody or an anti-sense RNA probe [2,9]. Molecular cloning of a full-length human prostasin cDNA revealed that its predicted amino acid residue sequence contains a carboxyl-terminal hydrophobic region that can potentially anchor the protein on the membrane [9]. At the amino acid level, prostasin is similar to plasma kallikrein, coagulation factor XI, hepsin, plasminogen, acrosin, prostase, and, in particular, testisin (sharing 44% sequence identity) [6,7,9]. A membrane-bound, *Xenopus* kidney epithelial cell sodium channel-activating protease (CAP1) was shown to be highly homologous to human prostasin as well (sharing 53% sequence identity at the amino acid level) [10]. Prostasin is encoded by a single-copied gene, which is located on human chromosome 16p11.2 [11].

The secreted prostasin cleaves synthetic substrates *in vitro* preferentially at the carboxyl-terminal side of Arg residue, and is thus considered a trypsin-like serine protease [2]. The physiological function of prostasin, however, has remained unknown. In an effort to define the physiological function of prostasin, we examined the expression of this protease in primary human prostate epithelial cells or human prostate cancer cell lines. Our findings indicated a potential down-regulation of prostasin in highly invasive prostate cancer cells. Three prostate cancer cell lines derived from the transgenic adenocarcinoma of the mouse prostate (TRAMP) model [12] were also shown to have null or reduced prostasin expression at the mRNA level. A down-regulation of prostasin protein expression was demonstrated in immunohistochemistry studies of prostate cancer tissue sections. These results implicate a function for prostasin serine protease in normal prostate, and its loss of expression may contribute to tumor progression. To test our hypothesis, we transfected invasive human prostate cancer cells with a

full-length human prostasin cDNA, and showed that a restoration of prostasin expression

reduces the invasiveness of the cancer cells *in vitro*.

MATERIALS AND METHODS

Cell Culture

All tissue culture media, sera, and supplements were purchased from LifeTechnologies (Gaithersburg, MD), except for those noted otherwise.

A normal human prostate epithelial cell primary culture (CC-2555) was obtained from Clonetics (San Diego, CA), and maintained in the supplied medium (Prostate Epithelial Cell Basal Medium, containing bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, gentamicin, and amphotericin). The culture was kept at 37°C with 5% CO_2 and used for experiments at the third passage.

Human prostate cancer cell lines LNCaP, DU-145, and PC-3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The PC-3 cells were maintained in F-12K medium supplemented with 10% fetal bovine serum (FBS) while the LNCaP and the DU-145 cells were maintained in RPMI-1640 medium supplemented with 10% FBS and 1 mM sodium pyruvate, and all were kept at 37°C with 5% CO_2.

The TRAMP-C1, C2, and C3 cell lines derived from the TRAMP model were cultured as previously described [12], in D-MEM with high glucose and L-glutamine, but without sodium pyruvate; supplemented with 5% Nu-serum (Collaborative Biochemical, Bedford, MA), 5% FBS, 5 µg/ml insulin, and 10^{-8} M dihydrotestosterone (DHT, Sigma-Aldrich Co., St. Louis, MO), at 37°C with 5% CO_2.

RNA Preparation and Analysis by RT-PCR-Southern Blot

Prostates of C57BL/6 mice (Harlan, Indianapolis, IN), or confluent cell cultures were used for total RNA extraction using the RNeasy kit from QIAGEN (Valencia, CA). The use of animals was approved by the IACUC of the University of Central Florida.

The human prostasin-specific RT-PCR (reverse transcription-polymerase chain reaction)/Southern blot analysis was performed as described previously [9]. One microgram of total RNA was used in the RT-PCR with two human prostasin gene-specific oligonucleotide primers [9], a Southern blot of the resolved RT-PCR samples was probed with a third prostasin gene-specific oligonucleotide [9], detecting an amplified 232-bp fragment.

The mouse prostasin-specific RT-PCR was performed using the following oligonucleotide primers: upstream, 5'-ATC ACC GGT GGT GGC AGT GC-3', downstream, 5'-TGG CTG CAG GGA GGC AGA TG-3'; which were derived from a mouse prostasin mRNA (cDNA) sequence (GenBank Accession Number: AI527990). This reported mouse prostasin cDNA sequence is 73% identical to the reported human prostasin cDNA sequence [9]. The sequence of the down-stream RT-PCR primer is complementary to both the human and mouse prostasin mRNA, while the upstream PCR primer is unique to the mouse prostasin mRNA. The PCR fragment that was amplified with these two primers is 342 bp in length. We probed the amplified PCR product with a full-length human prostasin cDNA to confirm the identity of the amplified PCR fragment as the mouse prostasin homologue. One microgram of RNA was used in the RT (at 37°C for 1 hr followed by 94°C for 5 min) and the PCR with the following program: 94°C/1 min – 60°C/1 min – 72°C/2 min for 30 cycles. One-fifth of the PCR product for each sample was resolved in a 0.8% agarose gel and Southern-transferred to an Immobilon-N membrane (Fisher Scientific, Pittsburgh, PA) and probed with a full-length human prostasin cDNA labeled by nick-translation using a reagent kit (LifeTechnologies). Following the hybridization, the membrane was washed to a final stringency of 1 x SSPE/0.1% SDS (1 x

SSPE: 0.18 M NaCl, 10 mM NaH$_2$PO$_4$, 1 mM Na$_2$EDTA, pH 7.4), at 60°C before an exposure of 30 min at -80°C to an X-ray film without an intensifying screen.

In both the human and mouse prostasin-specific RT-PCR, the following primers were used to co-amplify the β–actin mRNA for control of RNA quality and quantity: upstream, 5'-GAA CCC TAA GGC CAA CCG TG-3', downstream, 5'-TGG CAT AGA GGT CTT TAC GG-3'.

Tissue Specimens and Immunohistochemistry

Human prostate tissues were obtained from radical prostatectomies performed at the Florida Hospital South Orlando location, Orlando, FL; or the Orlando Regional Medical Center (ORMC), Orlando, FL. The procurement of human tissues was approved by the Institutional Review Boards (IRB) of Florida Hospital, ORMC, and the University of Central Florida. None of the subjects examined in our study underwent prior hormonal, radiation, or chemotherapy. Tissues were fixed in formalin, paraffin-embedded, and sectioned at 4 μm thickness. The sections used in our study contained both benign and neoplastic tissues, as evaluated upon hematoxylin/eosin staining of the adjacent sections.

Immunohistochemistry was performed according to Yu et al. [2] with some modifications. Briefly, the human prostate sections were de-paraffinized in Hemo-De (Fisher Scientific) followed by rehydration in a decreasing series of alcohol. The prostate sections were subjected to a standard antigen retrieval procedure (BD PharMingen, San Diego, CA) in citrate buffer, pH 6.0, followed by treatment in 3% H$_2$O$_2$. After blocking the sections with 10% goat serum (LifeTechnologies) in TBS-T (10 mM Tris-HCl, pH 7.6; and 150 mM NaCl, containing 0.1% Triton X-100), a prostasin-specific antibody [2] (diluted at a ratio of 1:500 in the blocking solution) was added for an incubation of 2 hours, followed by an incubation of 1 hr with the secondary antibody, goat anti-rabbit IgG (Sigma-Aldrich Co., used at 20 μg/ml). The sections

were then incubated with the peroxidase anti-peroxidase complex (Sigma-Aldrich Co., 1:200) for

1 hr. Sections were washed in TBS/0.1% Tween-20 between steps. The color reaction was

performed by incubating the sections with 3,3'-diaminobenzidine tetrahydrocholoride (0.5 mg/ml,

LifeTechnologies) and H_2O_2 (0.03%) for 15 min. The prostate sections were then counter-

stained with hematoxylin, dehydrated in an increasing series of alcohol and mounted with

Permount (Fisher Scientific). Control sections were treated with the same procedures as

described above, except, a pre-immune rabbit serum was used in place of the prostasin-specific

antiserum.

Evaluation of Immunohistochemical Staining

Sections from 39 radical prostatectomy specimens were subjected to prostasin

immunostaining. For each section used in prostasin immunostaining, an adjacent section was

stained with hematoxylin/eosin and evaluated by a pathologist (L.A.G.) to determine tumor

Gleason grades [13]. Prostasin immunostaining in each tumor area with a distinct Gleason

grade was recorded as a separate observation (n). For each case we selected 1 to 4 sections

to examine the different regions of the prostate from the patient. In total, 128 sections were

evaluated. Prostasin immunostaining in the prostate sections was evaluated by adopting, with

modifications, the scoring system used for HercepTest™ (DAKO Corporation, Carpinteria, CA).

A score of 0 is assigned to areas with no staining. A score of 1 (+) is assigned to areas with

faint epithelial staining and/or staining in fewer than 10% of cells. Scores 0 and 1 are defined as

negative for the data summarized in Table I. A score of 2 (++) is assigned to areas with

moderate epithelial staining in more than 10% of cells, and a score of 3 (+++) is assigned to

areas with strong staining in more than 10% of cells. Scores 2 and 3 are defined as positive for

the data summarized in Table I. Areas selected for scoring were viewed under 100 x

magnifying power. The differences in prostasin-specific staining mean scores between tumor grades were examined by analysis of variance (ANOVA, defining $p < 0.05$ as being significant).

Transfection of Cell Lines with Plasmid DNA and Selection of Stable Transfectants

A pREP-8 (Invitrogen, Carlsbad, CA) plasmid carrying a full-length human prostasin cDNA [9] was provided by Dr. Julie Chao of the Medical University of South Carolina (Charleston, SC). This plasmid was used to transfect the DU-145 cells. For transfection of the PC-3 cells, we re-engineered the plasmid as follows. The histidinol-resistance element of this human prostasin cDNA plasmid was removed via a Bgl II digestion (a 3,475-bp fragment), and replaced with a neomycin (G418)-resistance element, via an EcoR V-Dra I fragment (3,439-bp) of the pcDNA3 vector (Invitrogen). The resulting plasmid contains a full-length human prostasin cDNA under the control of the RSV promoter, a G418-resistance element, and the EBNA-1 element for episomal expression in primate cells. A similar drug-resistance gene rearrangement was made for the pREP-8 plasmid without the human prostasin cDNA, and the resulting plasmid was used as a vector control in our experiments. Drug-resistance genes were rearranged to make the new prostasin cDNA plasmid carry a G418-resistance element, because in our earlier experiments we found that the PC-3 cell line was not sensitive to the histidinol drug selection.

Transfection was carried out using a BTX-600 Electro-Cell-Manipulator (Gentronics, San Diego, CA), according to the recommended procedures. Briefly, 1,000,000 pelleted cells were re-suspended in 0.3 ml of the culture medium, and mixed with 50 µg of plasmid DNA dissolved in 0.1 ml of sterile distilled water. The cell/DNA mixture was then transferred to a 4-mm cuvette and pulsed at 200 volts, 1600 µF, 72 ohms, and 500V/Capacitance setting on the BTX-600. Following the pulse, cells remained in the cuvette at room temperature for 30 min before being transferred to a 25-cm^2 tissue culture flask containing the proper culture medium.

At 24 hr post electroporation, the culture medium was replaced with fresh medium containing either 5 mM histidinol (for DU-145 selection) or 800 µg/ml G418 (for PC-3 selection) and maintained in the presence of the drugs until colonies appeared (5-7 days). Colonies (100-200) were then dispersed *via* trypsinization, and maintained in the presence of drugs as a polyclonal culture without colony-isolation. Cells transfected with the human prostasin cDNA plasmids were assayed in western blot analysis for expression of the prostasin protein, using vector-transfected cells as negative control.

Western Blot Analysis

Cells grown to 80-100% confluence were washed three times in 1 x PBS (phosphate-buffered saline, pH7.4), and then lysed in TBS containing 1% Triton X-100 on ice for 30 min. The total lysates were centrifuged at 14,000 rpm for 30 min at 4°C to remove the pellet. Protein concentration was determined using a DC (detergent compatible) protein assay kit (Bio-Rad, Hercules, CA). The samples were then subjected to SDS-PAGE followed by western blot analysis using a prostasin-specific antibody [2]. Briefly, cell lysates were resolved in 10% gels under reducing conditions before electrotransfer to nitrocellulose (NC) membranes (Fisher Scientific). Upon complete protein transfer, the NC membranes were blocked in BLOTTO (5% non-fat milk made with TBS/0.1% Tween-20, pH7.6), incubated with the prostasin-specific antibody diluted at a ratio of 1:2,000 in BLOTTO, followed by an incubation with the secondary antibody, goat anti-rabbit IgG conjugated to HRP (horse-radish peroxidase) (Sigma-Aldrich Co.; used at 1:10,000). The bound secondary antibody was detected using enhanced-chemiluminescence (ECL) reagents (Pierce, Rockford, IL) according to the supplier's recommendations. All procedures were performed at room temperature, and the membranes were washed between steps.

Matrigel Chemoinvasion Assay

An *in vitro* Matrigel chemoinvasion assay was performed as follows. Basement membrane Matrigel stock (10 mg/ml, Collaborative Biochemical, Bedford, MA) was thawed overnight on ice in a refrigerator (0-4°C). Transwell invasion chambers (Costar, Cambridge, MA) with 8-μm pore polycarbonate filters (growth area 0.33 cm^2) were coated with a diluted Matrigel (50 μg/filter, a 1:3 dilution of the stock with chilled serum-free medium was used for coating). The gel was solidified by incubating the coated filters at 37°C for 60 min in a moist chamber. The lower chambers of the Transwell plates were filled with 0.6 ml serum-free medium, supplemented with 25 μg/ml fibronectin (Sigma-Aldrich Co.). Ten thousand cells in 0.1 ml serum-free OPTI-MEM I medium were placed onto each Matrigel-coated filter. The filter cartridge was then inserted into the lower chamber and the assembled plates were incubated at 37°C for 24 hr. After removing the medium, the filters were washed 3 times in 1 x PBS, fixed at room temperature for 20 min in 4% paraformaldehyde made with 0.1 M phosphate buffer (pH7.4), and then washed 3 times with the phosphate buffer. The filters were then stained with 1% toluidine blue (LabChem, Inc., Pittsburgh, PA) for 2 min. The cells on the Matrigel surface were removed with a Q-tip, and all cells on the underside of each filter were counted under a light microscope after mounting the filter on a glass slide. The invasion assays were done in triplicates per cell type for three times.

RESULTS

Prostasin Expression Is Absent in Invasive Prostate Cancer Cell Lines

Normal human prostate epithelial cells and three human prostate cancer cell lines were subjected to western blot analysis using a prostasin-specific antibody. As shown in Figure 1, upper panel, prostasin is detected as a 40-kDa band in normal human prostate epithelial cells (CC-2555) and in the non-invasive prostate cancer cell line LNCaP. The prostasin protein was not detected in two highly invasive human prostate cancer cell lines, DU-145 and PC-3, when the same amount of total cellular protein was analyzed.

By means of RT-PCR-Southern blot analysis, we demonstrated a corresponding difference of prostasin mRNA expression in these cells as well. Prostasin mRNA is detected in the CC-2555 and LNCaP cells, but not in the DU-145 or PC-3 cells (Fig. 1, middle panel). A co-amplification of a house-keeping gene, β-actin, message (Fig. 1, lower panel) was used as a control for the quantity and quality of the RNA used. The data indicate that prostasin expression is lost in highly invasive human prostate cancer cell lines.

Loss of prostasin expression was also seen in mouse prostate cancer cell lines. Since the human prostasin antibody does not cross-react with the mouse prostasin protein (data not shown), we examined mouse prostasin expression in the cell lines by RT-PCR/Southern blot hybridization with a human prostasin cDNA probe. Mouse prostasin mRNA was detected in normal mouse prostate, but was not detected in the TRAMP-C cell lines (Fig. 2, upper panel). Again, a co-amplification of a mouse house-keeping gene, β-actin, message was used as a control for RNA quantity and quality (Fig. 2, lower panel). A weak amplified mouse prostasin mRNA signal could be seen in the non-tumorigenic [12] TRAMP-C3 cells after a prolonged exposure, but not in the tumorigenic and invasive [12,14] TRAMP-C1 and TRAPM-C2 cells (data not shown).

Expression of Prostasin Protein Is Reduced in Human Prostate Cancer

Prostatectomy specimens from 39 patients (128 sections) were subjected to immunohistochemistry using a prostasin-specific antibody. Overall, in non-tumor or benign prostate epithelia, 89.0% of the examined areas demonstrated positive staining for prostasin protein and 11.0% were considered negative (see MATERIALS AND METHODS for scoring criteria). In all tumor specimens that were examined, prostasin was detected in 93.3% of the low Gleason grade areas ($\leq$ grade 2), 44.4% of Gleason grade 3 areas, and 15.4% of Gleason grade 4-5 areas (data summarized in Table I). The mean prostasin immunostaining score was found significantly decreased in high-grade prostate tumors as compared to that of non-tumor or low-grade (1-2) areas (ANOVA, $p < 0.0001$).

Representative staining images of non-tumor (benign) areas and prostate tumor areas are shown in Figure 3. The prostasin protein was detected in the cytoplasm and on the plasma membrane (apical) of benign epithelial cells lining the secretory lumen as well as in the secretion inside the lumen (Fig. 3A and 3B, score 3, or +++), confirming the results of Yu et al. [2]. When a pre-immune rabbit serum was used in place of the prostasin antiserum, no staining was observed in either the non-tumor epithelia (Fig. 3C and 3D) or tumor epithelia (Fig. 3E). Tumor epithelia displayed various degrees of prostasin immunostaining as shown in Figure 3F-3L. In Gleason grade 1-2 tumors, moderate prostasin staining is seen in the cytoplasm and on the plasma membrane of some epithelial cells, as well as in the secretion in the lumen (Fig. 3G and 3H, score 2, or ++). In Gleason grade 3 tumors, a lesser number of epithelial cells displayed the moderate level prostasin staining (Fig. 3I and 3J). In Gleason grade 4-5 tumors, most epithelial cells did not show any prostasin staining, while some prostasin staining can be seen in rare, sporadic tumor cells (Fig. 3K and 3L, as indicated by the arrow, score 0).

Re-expression of Human Prostasin Protein in Invasive Human Prostate Cancer Cells Reduces Invasiveness *In Vitro*

Following the electroporation and drug selection, 100-200 colonies formed for each transfected cell type. All colonies for each transfected cell type were kept in a mixed culture as polyclonal transfectants for the ensuing experiments. Polyclonal DU-145 and PC-3 cells transfected with the human prostasin cDNA (designated DU-145/Pro, and PC-3/Pro, respectively) were confirmed to express the human prostasin protein, as shown in the western blot analysis of the cell lysates (Fig. 4, upper panel). The vector-transfected cells, designated DU-145/Vector or PC-3/Vector, respectively, were used as negative control in the western blot. We further examined the DU-145/Pro and the PC-3/Pro cells by immunocytochemistry, and confirmed that 100% of the cells expressed the prostasin protein (data not shown).

In the *in vitro* Matrigel chemoinvasion assays (Fig. 4, lower panel), the invasiveness of DU-145/Pro cells was determined to be at 32% of that of DU-145/Vector cells (or, the reduction of invasiveness was at 68%). The invasiveness of PC-3/Pro cells was determined to be at 58% of that of PC-3/Vector cells (or, the reduction of invasiveness was at 42%).

We performed *in vitro* cell proliferation assays on DU-145/Pro *vs.* DU-145/Vector cells, and on PC-3/Pro *vs.* PC-3/Vector cells, but did not observe any difference between the growth rates of the prostasin cDNA-transfected or the vector-transfected cells over an 8-day period (data not shown).

DISCUSSION

Human prostasin serine protease was discovered in 1994 but its function was not defined [2]. We present evidence in this report to demonstrate prostasin as a potential invasion suppressor of prostate cancer.

In the prostate, prostasin is synthesized by the epithelial cells and may assume a membrane-bound form in these cells [2,9]. The demonstration of prostasin protein and mRNA expression in normal human prostate epithelial cells (Fig. 1) implicated that prostasin may have a function in normal prostate biology. On the other hand, a loss of prostasin expression in prostate cancer may also suggest a role for prostasin in suppressing prostate cancer progression. In the present study, the following results support our hypothesis: 1). Two highly invasive human prostate cancer cell lines, the DU-145 and the PC-3 [15], do not express prostasin at either the protein level or the mRNA level, while a non-invasive human prostate cancer cell line, the LNCaP [15], expresses this serine protease at both the mRNA and the protein levels (Fig. 1). 2). A loss of mouse prostasin mRNA expression was demonstrated in the tumorigenic and invasive [12,14] prostate cancer cell lines TRAMP-C1 and -C2 (Fig. 2). 3). Immunohistochemistry analysis of human prostate cancer demonstrated a down-regulated pattern of prostasin protein expression in high-grade prostate tumors (Fig. 3, and Table I).

Genetically, prostate tumors are heterogeneous and multi-focal in nature, in that one patient's gross-anatomy tumor comes from multiple initial lesions which are caused by different initial transformation events and progress to different stages by different ensuing transformations [16]. The Gleason grading, when used as a percentage of each cancer occupied by Gleason grade 4-5 areas, is independently associated with prostate cancer progression [17]. We demonstrated a significant decrease of prostasin expression in the high-grade, *i.e.*, the more progressively transformed tumors. In the case of mouse prostate adenocarcinomas in the TRAMP model, the tumors are induced by expression of the SV40 T-

antigen as an initial transformation event [18]. The TRAMP-C cell lines were established from a primary prostate tumor of a TRAMP mouse at 32 weeks of age, and represent different stages of the prostate epithelial cell tumor transformation process [12]. In this mouse prostate cancer cell line model, the more progressively transformed cells (TRAMP-C1 and –C2) did not express the prostasin mRNA (Fig. 2). A weak level of mouse prostasin mRNA expression was detected only in TRAMP-C3 cells following a prolonged exposure of the Southern blot in Figure 2 (data not shown), the significance of such a weak level of expression is presently unclear. In both the human prostate cancer cell lines and the primary tumors, as well as the mouse prostate cancer cell lines, however, the down-regulation of prostasin may be linked to the transformation stage and progression of prostate cancer.

An immediate step that could be taken to address prostasin's potential function in the prostate was to restore prostasin expression in the prostate cancer cell lines that lost expression. We used the human prostate cancer cell lines as a model to begin investigating prostasin's potential function. We forced the expression of prostasin in the DU-145 and PC-3 cells via transfection with a human prostasin cDNA. We found that a forced re-expression of the prostasin protein in DU-145 and PC-3 reduced the in vitro invasiveness of the cells by 68% and 42%, respectively (Fig. 4). We also tested our transfectants to determine if a forced re-expression of prostasin protein had any anti-proliferation effects on these cells grown in tissue culture. No significant difference was found between the growth rates of DU-145/Pro or PC-3/Pro cells and that of the vector-transfected cells, DU-145/Vector or PC-3/Vector (data not shown). This observation also validates the observed reduction of invasiveness in the invasion assay. We can attribute the reduced number of DU-145/Pro or PC-3/Pro cells invading through the Matrigel membrane to a change of their intrinsic invasive properties, not to a significantly slower growth rate over the assaying period. We further tested if the anti-invasion activity of prostasin may be attributed to the plasma membrane-bound, or to the secreted form of

prostasin by adding purified recombinant human prostasin (the secreted form, at 0.5 µM final concentration) in an invasion assay using the PC-3 cells. Our results indicated that the secreted prostasin did not reduce the invasiveness of PC-3 cells (data not shown).

CONCLUSIONS

The observation of down-regulation of prostasin expression in human and mouse prostate cancer suggests that prostasin serine protease may have a function in normal prostate, or play a role in suppressing prostate cancer progression. The *in vitro* anti-invasion activity of prostasin suggests that prostasin may be a potential invasion suppressor of prostate cancer.

ACKNOWLEDGMENTS

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Table I. Summary of Prostasin Immunostaining in Human Prostate Sections

		Tumor Gleason Grade		
	Benign	1-2	3	4-5
Total Areas Examined (n)	128	15	36	26
Positively Stained Areas (n)	114	14	16	4
Negatively Stained Areas (n)	14	1	20	22
Percentage of Positive Staining (%)	89.0	93.3	44.4	15.4
Mean Staining Score	2.024	1.933	0.889	0.308

Differences in prostasin staining intensity were examined by analysis of variance (ANOVA). The expression of prostasin was significantly decreased in high-grade prostate tumor as compared with benign prostate or low-grade prostate tumor ($p < 0.0001$).

FIGURE LEGENDS

Fig. 1. Human prostasin expression in prostate epithelial cells. By means of western blot analysis (upper panel), prostasin (as a 40-kDa band) was detected in normal human prostate epithelial cells (CC-2555) and the LNCaP cells, but not in the DU-145 or PC-3 cells. An equal amount of total protein (100 μg) was loaded for each sample. At the mRNA level, human prostasin mRNA (*via* a 232-bp amplified DNA band) was detected in normal prostate epithelial cells (CC-2555) and the LNCaP cells, but not in the DU145 or PC-3 cells as analyzed by RT-PCR/Southern blot hybridization (middle panel). Co-amplification of a 556-bp human β–actin message (as shown in the gel photograph in the lower panel) confirmed the quality and the quantity of the RNA applied in each RT-PCR.

Fig. 2. Mouse prostasin mRNA expression. Total RNA (1 μg) from a normal mouse prostate (whole), and the TRAMP-C cells were subjected to an RT-PCR using two mouse prostasin-specific oligonucleotide primers (see MATERIALS AND METHODS). The amplified mouse prostasin message (a cDNA band of 342 bp) was then probed with a full-length human prostasin cDNA, as shown in the autoradiogram in the upper panel. Note the strong signal of mouse prostasin message in the normal prostate RNA sample but not in RNA samples of the TRAMP-C cells. Co-amplification of a 560-bp mouse β–actin message (as shown in the gel photograph in the lower panel) confirmed the quality and the quantity of the RNA applied in each RT-PCR.

Fig. 3. Immunoperoxidase staining of prostasin in human prostate. Paraffin-embedded human prostate sections were stained for prostasin protein expression evaluation using a specific antibody [2] as described in MATERIALS AND METHODS. Prostasin positive staining (brown

color) was detected in the cytoplasm and apical membrane in non-tumor or benign epithelial cells (image A, or B: the boxed area of A). When prostasin antibody was omitted in the immunohistochemistry procedures, neither non-tumor (image C, or D: the boxed area of C), nor tumor epithelial cells (image E) displayed any staining. Tumor epithelial cells showed reduced prostasin staining (image F) as compared to non-tumor epithelial cells in adjacent areas (image F). Representative areas, in which prostasin staining is reduced in tumor epithelial cells are shown in image G, or H: the boxed area of G (Gleason grade 2); image I, or J: the boxed area of I (Gleason grade 3), and image K, or L: the boxed area of K (Gleason grade 4). Magnification: A, C, E, I, and K: 25X; G: 100X; B, D, F, H, J and L: 200X.

Fig. 4. Recombinant prostasin protein expression and *in vitro* invasive properties of the DU-145 and the PC-3 transfectants. DU-145 or PC-3 cells transfected with either a vector DNA (labeled as "vector") or a prostasin cDNA construct (labeled as "prostasin") were analyzed by a western blot analysis using a prostasin-specific antibody (upper panel) or subjected to an *in vitro* Matrigel chemoinvasion assay (lower panel) as described in MATERIALS AND METHODS. The expressed recombinant human prostasin protein (a 40-kDa band) was detected in the prostasin cDNA-transfected DU-145 or PC-3 cells, but not in the vector-transfected cells. In the Matrigel chemoinvasion assay, the vector-transfected cells are expressed as being 100% invasive (solid bars), the open bars represent the relative invasiveness of the human prostasin cDNA-transfected cells. The data were analyzed by a Student *t*-test using the StatView software (Abacus Concepts, Inc., Berkeley, CA).
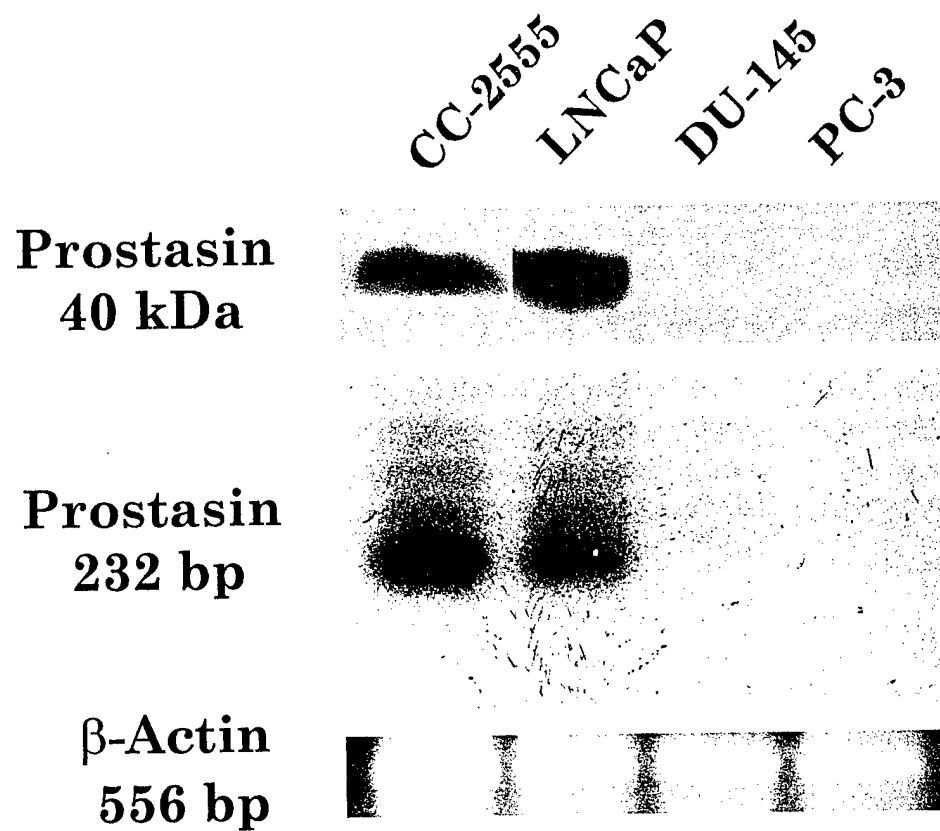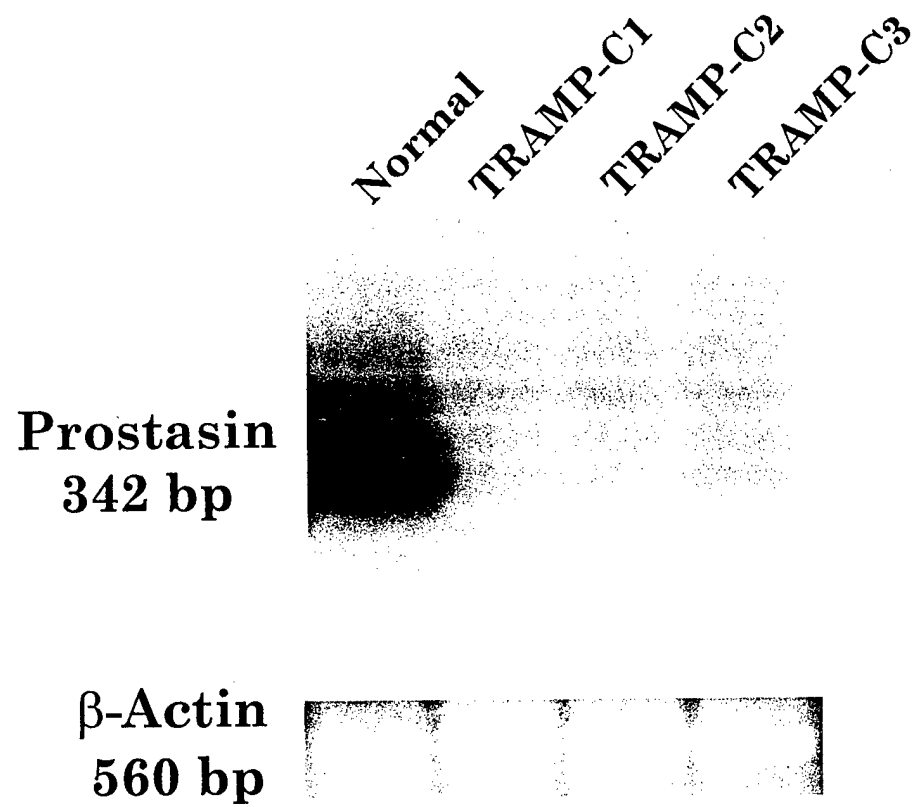
Figure 1

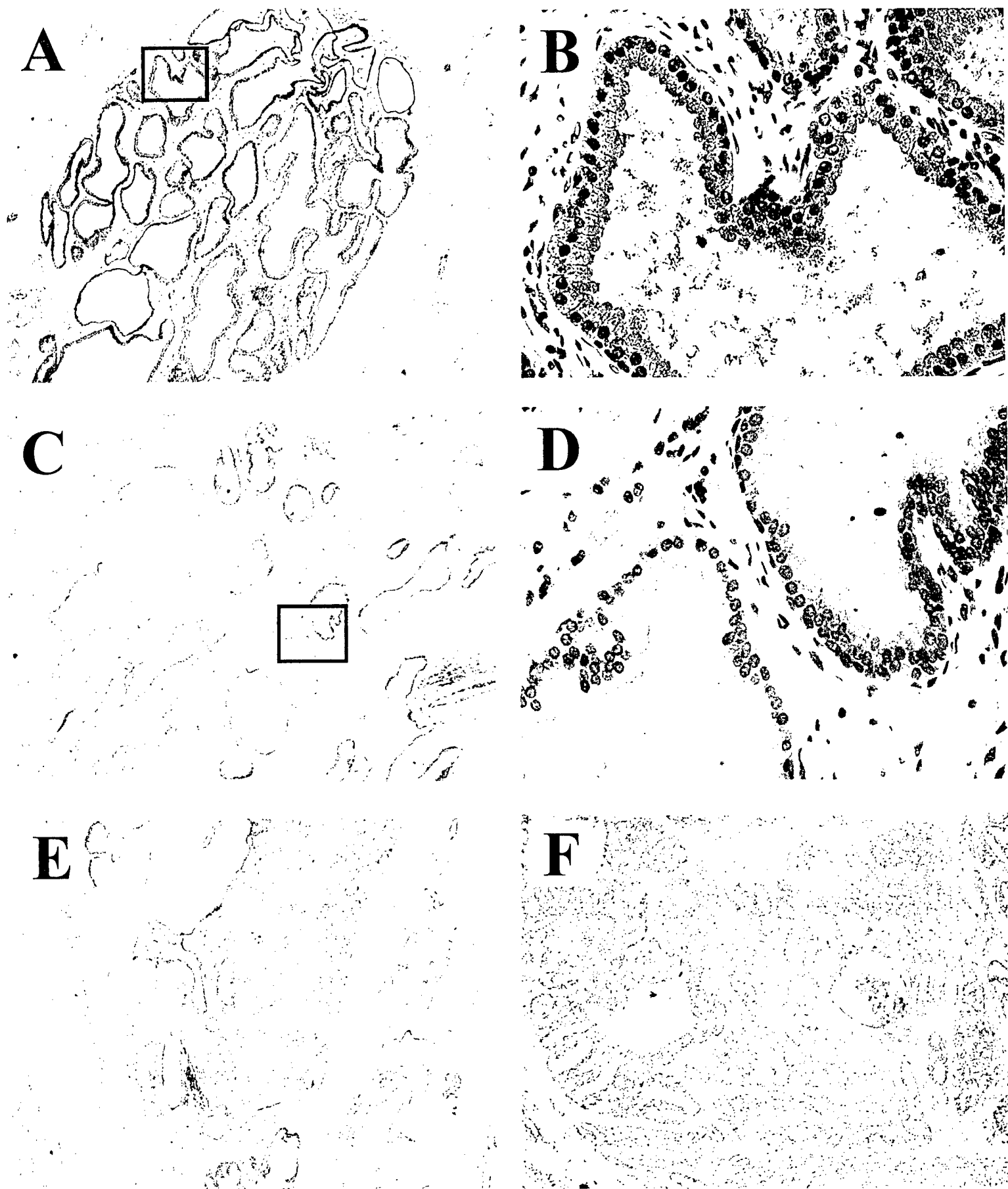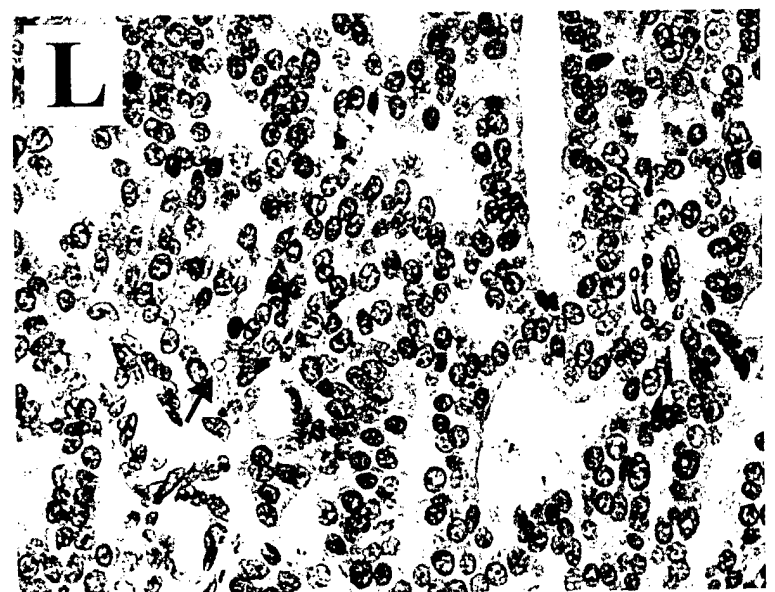**Prostasin 342 bp**

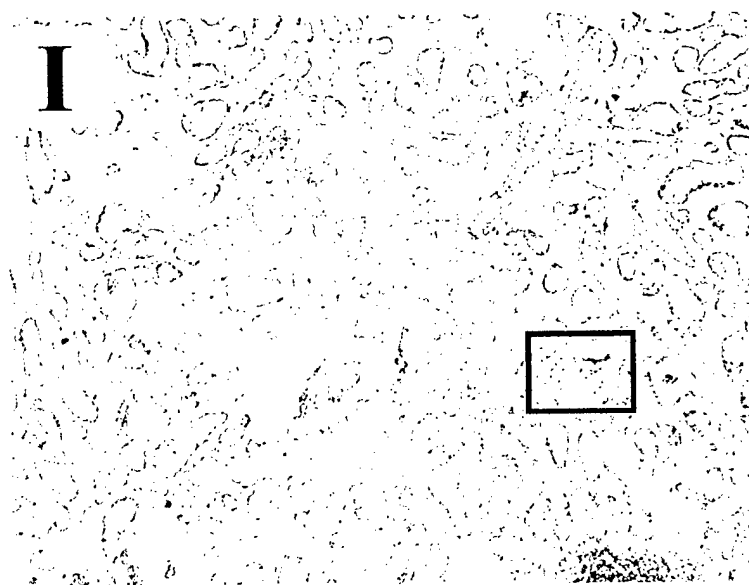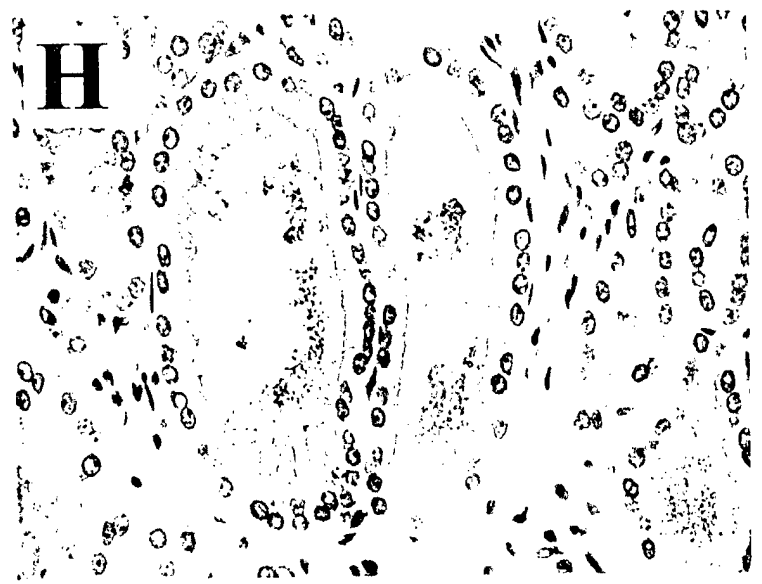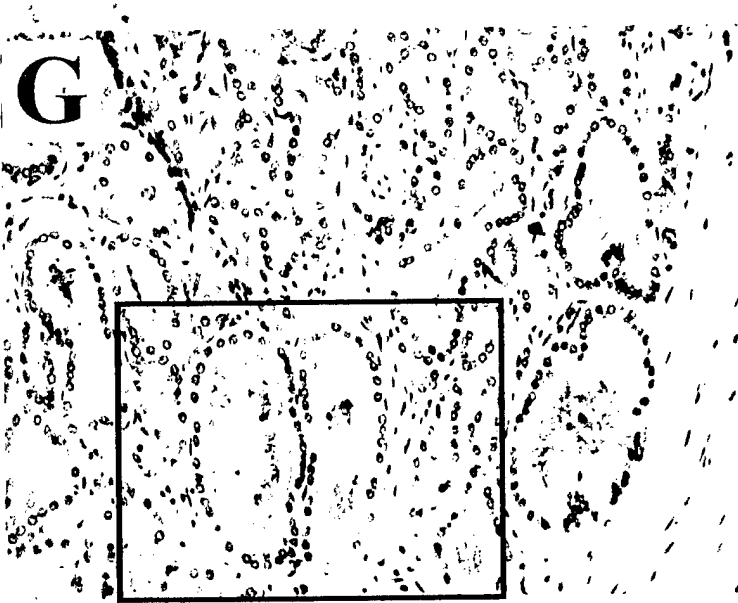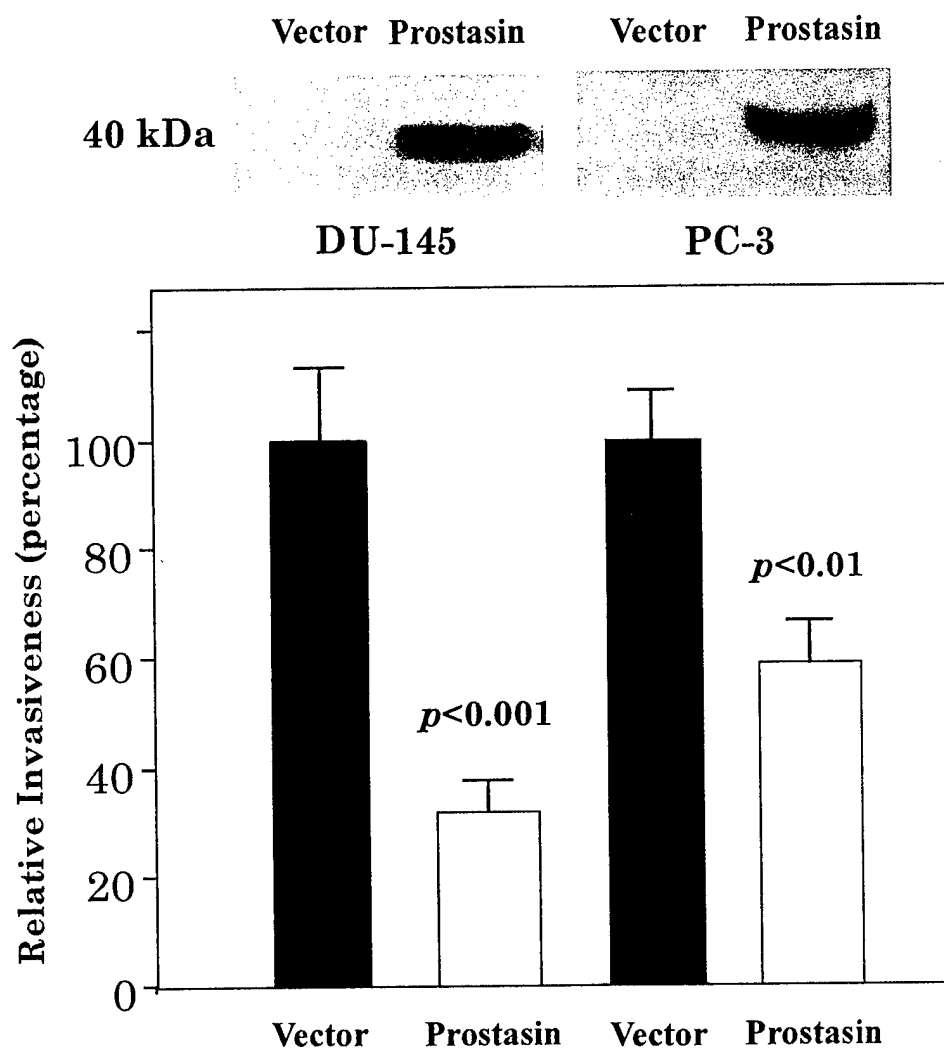**β-Actin 560 bp**
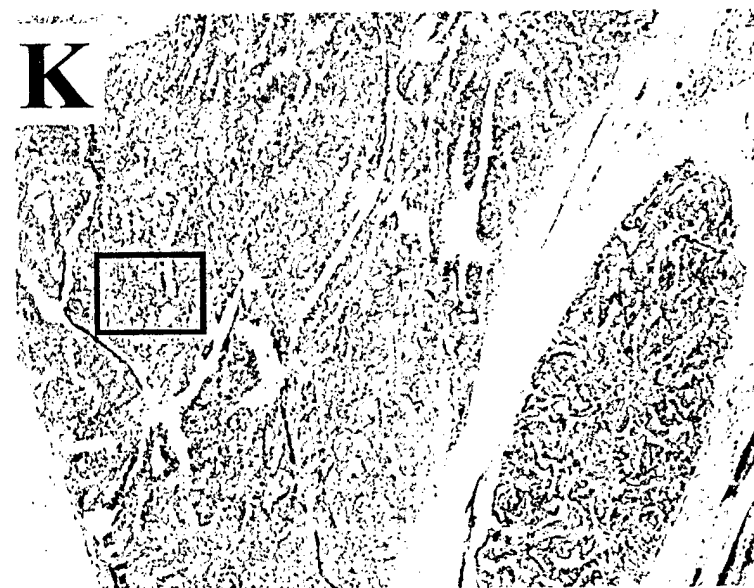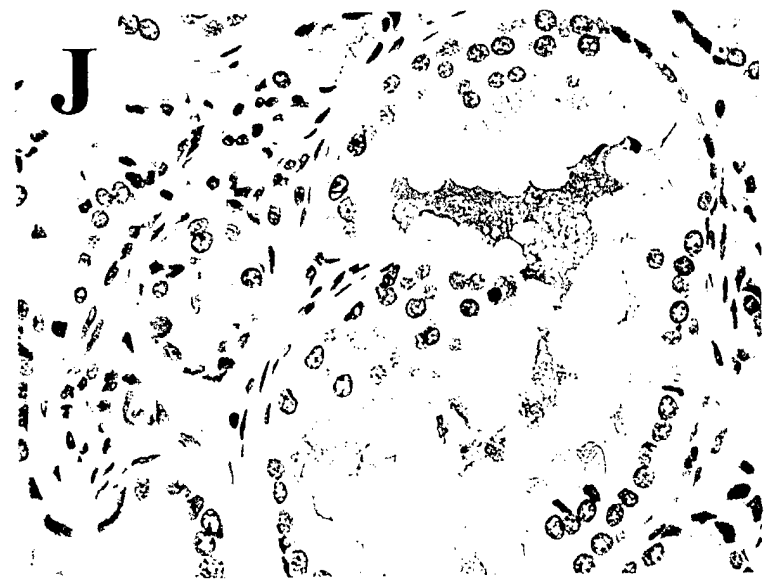
Figure 2

Figure 3

Figure 3 Continued

Figure 4

Prostasin Is A Glycosylphosphatidylinositol-anchored Active Serine Protease

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1

Summary

A recombinant human prostasin serine protease (r-hPro) was expressed in several human cell lines. Subcellular fractionation showed that r-hPro is synthesized as a membrane-bound protein, while a free-form prostasin is secreted into the culture medium. Prostasin was identified in nuclear and membrane fractions. Membrane-bound prostasin can be released by phosphatidylinositol-specific phospholipase C treatment, or labeled by [^{3}H]ethanolamine, indicating a glycosylphosphatidylinositol-anchorage. A prostasin-binding protein was identified in mouse and human seminal vesicle fluid. Both the secreted and the membrane-bound prostasin were able to form a covalently linked 82-kDa complex when incubated with seminal vesicle fluid. The complex formation between prostasin and the prostasin-binding protein was inhibited by a prostasin antibody, heparin, and serine protease inhibitors. Prostasin's serine protease activity was inhibited when bound to the prostasin-binding protein in mouse seminal vesicle fluid. This study indicates that prostasin is an active serine protease in its membrane-bound form.

Introduction

Prostasin is a serine protease discovered in ejaculated human semen in 1994 (1). The molecular mass of prostasin is 40 kDa when examined by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Prostasin displays trypsin-like enzymatic activities by hydrolyzing peptidyl fluorogenic substrates such as D-Pro-Phe-Arg-AMC. This trypsin-like enzymatic activity can be inhibited by aprotinin, antipain, leupeptin, and benzamidine. Prostasin is present at high levels in normal human semen (8.61 ± 0.42 μg/ml) and in the prostate gland (143.7 ± 15.9 ng/mg). Lower amounts of prostasin can also be detected in other tissues. In the prostate gland, the prostasin protein is present in the epithelial cells as well as in the secretion inside the lumen. The full-length human prostasin mRNA has been deduced (2). The predicted mature prostasin peptide sequence has a potential carboxyl-terminal hydrophobic membrane-anchorage domain followed by a short cytoplasmic tail. The translated amino acid residue sequence of prostasin is similar to those of human prostase, testisin, plasma kallikrein, coagulation factor XI, hepsin, plasminogen, and acrosin (2-4). A membrane-bound, *Xenopus* kidney epithelial cell sodium channel-activating protease (CAP1) was found highly homologous to human prostasin, sharing 53% sequence identity at the amino acid level (5). Recently, the mouse counterpart of CAP1, mCAP1, has been cloned from a cortical collecting duct cell line (6). The mCAP1 shares 77% amino acid sequence identity with human prostasin.

Serine proteases play important roles in a diverse range of the body's normal physiological processes, and are implicated in various pathological processes such as cardiovascular disorders and cancers (7). The prostate produces a number of serine proteases such as the prostate-specific antigen (PSA) (8), human glandular kallikrein (hK2) (9), and the most recently discovered prostase (3). Some of these serine proteases are suspected to affect fertility or semen liquefaction (10), while others are implicated in normal prostate development or prostatic diseases (11-14). For example, both PSA and hK2 have become important diagnostic and prognostic markers for prostate cancer. Serine proteases are usually regulated at the post-translational level, in addition to the transcriptional regulation at their gene level. The body's own strategy of regulating the serine proteases is to bind the serine proteases with a protein inhibitor, such as the inhibitors of the serpin class (15). These serpin-serine protease pairs are highly specific with regard to the two molecules involved, examples include α_1-antitrypsin/elastase (15), kallistatin/kallikrein (16, 17), and α_1-antichymotrypsin/PSA (18). The mechanism of serpin inhibition of serine proteases involves the formation of a covalently-linked

complex at a 1:1 stoichiometry (19). Such complex exhibits resistance to treatment with SDS, or boiling (16, 17).

Prostasin's physiological functions are not fully understood. In a recent study (20), we have shown that prostasin expression is significantly down-regulated in high-grade prostate tumors and lost in highly invasive human and mouse prostate cancer cell lines. Transfection of two human prostate cancer cell lines DU-145 and PC-3 with human prostasin cDNA reduced *in vitro* invasiveness of the cells, suggesting an invasion suppressor role for prostasin. This anti-invasion activity is apparently conferred by the cellular prostasin, but not the secreted prostasin. In the present study, we determined that prostasin is a GPI-anchored membrane protein in addition to being a secreted protease. Prostasin's subcellular localization was investigated in cells expressing native or recombinant prostasin. We have also identified a prostasin-binding protein, a potentially serpin-class serine protease inhibitor specific for prostasin. We further demonstrated that the membrane-bound prostasin is an active serine protease. These results will provide structural and regulatory information for further investigation of the functions of prostasin in normal prostate development, prostatic diseases, as well as reproductive biology.

Experimental Procedures

Cell Lines and Plasmid DNA Transfection

A human embryonic kidney epithelial cell line, 293-EBNA (Invitrogen, Carlsbad, CA), was maintained in Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% fetal bovine serum (FBS). Human prostate cancer cell lines LNCaP, DU-145, and PC-3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The LNCaP and the DU-145 cells were maintained in RPMI-1640 medium supplemented with 10% FBS and 1 mM sodium pyruvate while the PC-3 cells were maintained in F-12K medium supplemented with 10% FBS. All cells were kept at 37°C with 5% CO_2. All tissue culture media, sera, and supplements were purchased from LifeTechnologies (Gaithersburg, MD).

A full-length human prostasin cDNA of 1896 base pairs (bp) (including a 209-bp 5'UTR, 1032-bp of the coding region, and a 655-bp 3'UTR) was generated by means of reverse-transcription-polymerase chain reaction (RT-PCR) with the following two primers, 5'-AGA CGG TGC TGG TGA CTC GT-3' and 5'-TGT GCT CAA ACA TTT TAA TC-3', using the total RNA of LNCaP cells as template (2). The amplified cDNA was cloned into a mammalian expression vector, pREP-8 (Invitrogen), at its polylinker site. Transfection of the prostasin cDNA plasmid into 293-EBNA cells was carried out *via* electroporation. The electroporated cells were then subcultured for selection of transfectants (293/Pro) using 5 mM histidinol (Sigma, St. Louis, MO) in the culture medium for 2 weeks. The pREP-8 vector plasmid was transfected into 293-EBNA cells and subjected to histidinol selection to establish the control cells (293/Vec).

The DU-145 and the PC-3 cells, which do not express prostasin (20), were also transfected using plasmids containing the full-length human prostasin cDNA. The methods for plasmid engineering and establishment of transfectants that express human prostasin were described previously (20). The resulting cell lines that express human prostasin were designated DU-145/Pro and PC-3/Pro.

SDS-PAGE and Western Blot Analysis

These procedures were carried out for all experiments unless stated otherwise. Samples were suspended in 1 x SDS sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% v/v glycerol; 2% w/v SDS; and 2% ß-mercaptoethanol), boiled for 5 min, and resolved in a 10% polyacrylamide gel. The resolved proteins were then transferred to a nitrocellulose membrane. The membrane was stained with India ink for 15 min (1:1000 in TBS-T: 20 mM Tris, pH 7.6;

5

containing 0.137 M NaCl and 0.1% Tween-20), blocked in 5% non-fat milk for 1 h, and incubated with the primary antibody for 30 min in a tray or a Surf-blot apparatus (Idea Scientific, Inc., Minneapolis, MN). After washing, the membrane was incubated with a secondary antibody conjugated with HRP (Sigma, used at a 1:10,000 dilution) for 30 min. Signals were detected using an ECL (enhanced chemiluminescence) detection procedure with the WestPico reagents (Pierce, Rockford, IL) following the supplier's protocol. The membrane was then exposed to Kodak X-ray film. The primary antibodies used were as follows: polyclonal antibodies against prostasin (recombinant or native, used at 1:1000), a monoclonal antibody against β1-integrin (used at 1:1000), and a monoclonal antibody against poly(ADP-ribose) polymerase (PARP) (used at 1:500). Antibodies against β1-integrin and PARP were from BD Transduction Laboratories (San Diego, CA).

Purification of Recombinant Human Prostasin

The 293/Pro cells were grown to a confluent monolayer in D-MEM/10%FBS containing 5 mM histidinol. Cells were then placed in OPTI-MEM I serum-free medium (LifeTechnologies) for 72 h before collection of the conditioned medium. The collected medium was tested for recombinant prostasin (r-hPro) by western blot analysis using a prostasin-specific antibody (1). For purification of the secreted prostasin, the serum-free medium was centrifuged at 10,000 rpm for 20 min to remove dead cells or debris, and then passed through an aprotinin-agarose column (1.5 x 20 cm, Sigma) equilibrated with 25 mM Tris-HCl, pH 7.6, at a flow rate of 25 ml/h. After extensive washing to remove any unbound proteins, the bound-prostasin was eluted with 0.1 M glycine (pH 3.0) containing 0.1 M NaCl, at a flow rate of 60 ml/h. The eluted prostasin was immediately neutralized with appropriate amounts of 1 M Tris-base, combined, concentrated with Centricon-10 concentrators (Amicon Inc., Beverly, MA) and stored at −20°C before use in other assays.

Preparation of A Polyclonal Antiserum against Recombinant Prostasin

The purified r-hPro (250 µg) in 0.5 ml PBS (phosphate-buffered saline, pH 7.4) was emulsified with an equal volume of complete Freund's adjuvant (Sigma) and was injected subcutaneously into a 1.5-kg female New Zealand White rabbit (Charles River Laboratories, Wilmington, MA). Booster injections with 100 µg of r-hPro (emulsified with incomplete Freund's

adjuvant, Sigma) were performed 3 times at 3-week intervals. Pre-immune rabbit serum was collected before the initial immunization.

Immunocytochemistry

The PC-3/Pro or LNCaP cells were seeded on glass coverslips (Fisher Scientific, Pittsburgh, PA) at a density of 5×10^4/coverslip and grown for 24-36 hours prior to a double-immunostaining. Briefly, cells were rinsed in 1 x PBS for 3 times, fixed in 4% paraformaldehyde, and permeablized with 0.18% Triton X-100 in PBS for 10 min. After blocking in 10% normal goat serum (LifeTechnologies) in 1 x PBS, cells were incubated with the primary antibodies for 45 min, washed and followed by incubation with the appropriate secondary antibodies at room temperature for 30 min, and then washed three times for 10-min/each in 1 x PBS. A rabbit polyclonal antibody against prostasin was used at a dilution of 1:100. A monoclonal antibody against PARP was used as a nuclear-specific marker at a dilution of 1:75. A goat anti-rabbit IgG-conjugated with fluorescein (FITC) (1:50, LifeTechnologies) and a goat anti-mouse IgG-conjugated with Cy3 (1:800, Jackson ImmunoResearch, West Grove, PA) were used as the secondary antibodies. The coverslips were mounted with Gel/Mount (Fisher Scientific) and analyzed on a Carl-Zeiss LSM510 laser scanning microscope.

Subcellular Fractionation and Differential Extraction

Subcellular fractionation was performed as described in Krajewske et al. (21) and Pemberton et al. (22). Briefly, confluent cells in 4×150-cm^2 flasks (estimated $5-10 \times 10^7$ cells/total) were washed with 1 x PBS three times, and removed by mechanical force for the 293/Pro cells, or trypsin treatment (0.25% with 1 mM EDTA) for the PC-3/Pro and LNCaP cells. The cells were resuspended in 7 ml of cold MES buffer (17 mM morpholinopropanesulfonic acid, pH 7.4; 2.5 mM EDTA; 250 mM sucrose) containing protease inhibitors (1 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, and 2 µg/ml antipain). The following steps were performed at 4°C. Cell suspension was homogenized with a Dounce homogenizer for 60 strokes followed by a centrifugation at 500 x g for 10 min twice, resulting in the crude nuclear fraction in the pellet. The supernatant was centrifuged at 10,000 x g for 15 min twice, resulting in the heavy membrane fraction in the pellet containing mitochondria, lysosomes, and peroxisomes. The supernatant from the 10,000 x g centrifugation was subjected to an ultra-centrifugation at 100,000 x g for 60 min, resulting in a light membrane fraction in the pellet containing the plasma

7

membrane, microsomes, and endoplasmic reticula. The supernatant from the final centrifugation contains soluble or cytosolic proteins. The pellets from each centrifugation were washed with 2 x 10 ml of MES to eliminate carry-overs.

Differential extraction of membrane fractions was carried out according to Pei et al. (23). Briefly, pellet/membrane fractions were divided equally into three portions and were extracted with 1% Triton X-114 in Tris buffer (10 mM Tris-HCl, pH 7.5), or high salt (350 mM NaCl in Tris buffer), or alkali (50 mM glycine/NaOH, pH 11.0) for 1 hr on ice. The samples were centrifuged at 100,000 x g for 30 min. The resulting pellet was dissolved in 1 x SDS sample buffer for gel electrophoresis. The supernatant was subjected to a TCA (trichloroacetic acid) precipitation to recover proteins for gel electrophoresis.

Detergent Phase Separation and PI-PLC Treatment

The procedure was adapted from those described by Bordier (24) and Rosenberg (25). Briefly, cells (5 x 10^6) were lysed in 1 ml ice-cold TBS (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA) containing 1% Triton X-114 (Sigma) and protease inhibitors, for 2 h with gentle shaking at 4°C. The lysate was then centrifuged at 14,000 rpm for 30 min. The supernatant (500 µl, or 700-800 µg of total protein) was overlaid onto a 300-µl sucrose cushion (6% w/v sucrose in TBS containing 0.06% Triton X-114). The solution was incubated at 37°C for 3 min and centrifuged at 300 x g for 3 min at room temperature to separate the detergent phase (pellet) and the aqueous phase. The aqueous phase was removed and further extracted with 0.5% Triton X-114 and 2% Triton X-114. The aqueous phase after the final centrifugation contains the soluble proteins. The detergent phase (pellet) from the first centrifugation was resuspended in 500 µl of ice-cold TBS, incubated at 37°C for 3 min, and centrifuged at 300 x g for 3 min at room temperature to ensure the purity of the detergent phase.

The detergent phase was resuspended in 100 µl of ice-cold TBS. Ten microliters of the resuspended detergent phase were subjected to phosphatidylinositol-specific phospholipase C (PI-PLC, Sigma) digestion at 37°C for 1 h with gentle shaking in a total volume of 100 µl reaction buffer (10 mM Tris-HCl, pH 7.5; 144 mM NaCl). One hundred microliters of ice-cold TBS containing 2% Triton X-114 were then added to the digestion mixture and subjected to phase separation as described above. At the final step, both the aqueous and detergent phases were precipitated with 6% w/v TCA and 0.013% sodium deoxycholate. The precipitates

were resuspended in 30 µl of 1 x SDS sample buffer, neutralized with ammonium hydroxide (microliter amounts), boiled and subjected to SDS-PAGE and western blot analysis.

Human prostates removed by radical prostatectomy performed at Orlando Regional Medical Center (ORMC, Orlando, FL) were sectioned with a Cryostat at 20-µm thickness. Eighty sections were collected and rinsed with PBS twice to remove prostatic fluid. The washed prostate sections were lysed in 1 ml TBS containing 1% Triton X-114 at 4°C overnight with rocking. The lysed prostate tissues were centrifuged and subjected to the same phase separation and PI-PLC treatment procedures as described above. Several representative prostate sections (7 µm) cut at intervals of the eighty 20-µm sections were subjected to standard hematoxylin/eosin staining for confirmation of benign prostate morphology. The use of human tissues was approved by the Institutional Review Boards (IRB) of ORMC and the University of Central Florida.

[³H]Ethanolamine Labeling, Immunoprecipation, and Fluorography

PC-3/Pro (3 x 10⁵) or LNCaP (1 x 10⁶) cells were seeded in a 35-mm dish in 1 ml OPTI-MEM I serum-free medium. Next day, [1-³H]ethan-1-ol-2-amine hydrochloride (37 MBq, 1mCi/ml, and 30.0Ci/mmol, Amersham Pharmacia Biotech, Piscataway, NJ) was added to the culture medium at a concentration of 100 µCi/ml, and the cells were cultured for another 24 h. Cells were washed once with PBS and lysed in 0.5 ml of RIPA buffer (PBS, pH 7.4; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS) containing protease inhibitors at 4°C for 1 h. The lysate was centrifuged at 14,000 rpm for 30 min to remove insoluble material. The supernatant was subjected to immunoprecipitation with 2 µg/ml anti-prostasin IgG (purified using Econo-Pac Protein A cartridge, Bio-Rad, Hercules, CA) and Protein A Sepharose beads (Sigma) at 4°C overnight. The beads were washed with RIPA buffer three times, resuspended in 2 x SDS sample buffer with β-mercaptoethanol, boiled and analyzed by SDS-PAGE. The gel was fixed in a solution of 2-propanol : water : acetic acid (12.5 : 32.5 : 5) for 30 min and soaked in Amplify fluorographic reagent (Amersham Pharmacia Biotech) for another 30 min. The gel was dried, and [³H]-labeled molecules were detected by exposure to an X-ray film with an intensifying screen at –80°C for 14 days.

Prostasin-binding Assay

Seminal vesicle fluid was expressed from one pair of mouse seminal vesicles (C57BL/6 mouse, Harlan, Indianapolis, IN) and mixed with 1 ml of 25 mM Tris-HCl, pH 7.6; and centrifuged at 14,000 rpm at 4°C for 30 min. Five microliters of the supernatant (50 μg total protein) were incubated with either the purified recombinant human prostasin (0.5 μg), or subcellular fractions of 293/Pro and PC-3/Pro cells (prepared in the absence of serine protease inhibitors) at 37°C for 60 min, or for various time periods for a time-course study. The binding reaction was stopped by the addition of SDS sample buffer and heating at 100°C for 5 min. Mouse tissues were homogenized in PBS (1 gram tissue/5 ml) and centrifuged in a microcentrifuge at 14,000 rpm for 30 min at 4°C. Forty micrograms of total protein for each tissue extract were used in the binding assay. Human or mouse plasma (1 μl), was also subjected to prostasin-binding assay. The use of animals was approved by the IACUC of the University of Central Florida. Human seminal vesicles were obtained from radical prostatectomy performed at ORMC. No seminal vesicle metastasis from prostate cancer was found according to the pathology report. Human seminal vesicle fluid was diluted with PBS at a ratio of 1:2, mixed by vortex and spun. Ten microliters of the diluted fluid were incubated with purified prostasin (0.5 μg) at 37°C for 60 min.

Membrane Overlay Zymography

The membrane overlay zymography was carried out using the protocols of Enzyme System Product (Livermore, CA) and Beals et al. (26). Briefly, samples were first resolved in a 10% polyacrylamide gel without SDS or β-mercaptoethanol. Following electrophoresis, the gel was equilibrated in a reaction buffer (50 mM Tris-HCl, pH 9.0) for 15 min. Pre-wet acetate cellulose membrane impregnated with the prostasin substrate D-Pro-Phe-Arg-AFC (Enzyme System Product) was then carefully laid over the gel without entrapping air bubbles. The membrane-overlaid gel was placed in a moist chamber at 37°C for 3-5 hr. The reaction was monitored using an ultraviolet (UV) lamp and photographed.

Results

Expression and Purification of Recombinant Human Prostasin

Serum-free conditioned medium from 293/Pro cell culture was prepared and passed through an aprotinin-agarose column for a one-step affinity-chromatographic purification of the recombinant prostasin as described in Experimental Procedures (also see Ref. 1). A Coomassie blue staining of the purified recombinant prostasin is shown in Figure 1, left panel (r-hPro). The r-hPro migrates at 40 kDa on an SDS-PAGE under reducing conditions. Due to glycosylation of the prostasin molecule (1, 2), it appeared as a rather diffused band on the gel. We prepared a polyclonal antibody (r-Pro Ab) using the purified r-hPro as an antigen. The r-Pro Ab recognized the purified recombinant prostasin (secreted form), the recombinant prostasin in 293/Pro total cell lysate (non-secreted form), and the native prostasin in ejaculated human semen (obtained from healthy volunteers, Ref. 1) (Figure 1, lanes 1, 2, and 4, upper right panel). The prostasin protein in the same set of samples was also recognized by a prostasin-specific antibody previously referenced (1) (Figure 1, lower right panel). Neither antibody cross-reacts with any non-specific protein in the control 293/Vec total cell lysate (Figure 1, right panel, lanes 3). The results indicate that the polyclonal antibody against the recombinant prostasin is specific to prostasin, and that the recombinant prostasin prepared using the amplified cDNA has a similar immunological reactivity as the native prostasin. The antibody against the recombinant prostasin was used in the ensuing assays conducted in this study.

Recombinant Prostasin Is A Membrane-bound Protein

A hydropathy plot of the translated prostasin amino acid sequence indicated that the prostasin polypeptide has a potential membrane-anchorage domain at the carboxyl terminus (2). To confirm the presence of a potentially membrane-anchored form of the prostasin protein, the 293/Pro cells were subjected to subcellular fractionation by differential centrifugation. Figure 2 shows that prostasin was present in the crude nuclear fraction (P1, 500-g pellet), heavy membranes (P2, 10,000-g pellet), and light membranes (P3, 100,000-g pellet), as determined by western blot analysis. Prostasin was also detected in the cytosol (S). The purified r-hPro was used as positive control. Equal amounts of total protein (30 μg) from each membrane fraction and the cytosol were applied in each lane.

In order to show that the membrane-anchored prostasin is not a peculiarity in the 293 cells, we performed similar subcellular fractionation analysis on prostasin cDNA-transfected

human prostate cancer cell line PC-3 (PC-3/Pro) and the human prostate cancer cell line LNCaP, which expresses endogenous prostasin (2, 20). As shown in Figure 3A, prostasin is detected in P1, P2, and P3 fractions, but not in the cytosol (S) of PC-3/Pro. In the LNCaP cells, endogenously expressed prostasin is detected only in the P3 fraction. The membrane fractions from PC-3/Pro cells were then immunoblotted with a monoclonal antibody against a nuclear protein PARP or a monoclonal antibody against a plasma membrane-bound protein β1-integrin, to ensure the purity of each fraction. The results showed that the prostasin protein exists in a membrane-bound form in all cell lines tested. The cells transfected with the vector DNA alone (293/Vec and PC-3/Vec) were subjected to the same fractionation procedures followed by SDS-PAGE/western blot analysis. No prostasin was detected (data not shown). We further subjected PC-3/Pro and LNCaP cells to a double-immunostaining and analyzed prostasin's subcellular localization using confocal microscopy. The confocal microscopic analysis of PC-3/Pro cells localized prostasin (green) to the ER/Golgi complex (Figure 3B), consistent with the cell fractionation results shown in Figure 3A. Since the nuclear membrane is practically a prominent component of the ER (27), it is not surprising that this portion of prostasin appeared in the P1 fraction. The LNCaP cells, however, did not show punctate or nuclear/ER/Golgi complex staining, again, consistent with the cell fractionation results shown in Figure 3A.

To test if prostasin is truly a membrane-anchored protein, rather than a membrane-associated protein, we subjected the P1, P2, and P3 fractions of the PC-3/Pro cells to treatment with a detergent, high salt, or alkali. As shown in Figure 4, membrane-bound prostasin (pellet) was released into the supernatant only by the detergent treatment (TX), but not the high salt (HiS) or alkali treatment (Alk). The detergent released prostasin and the membrane-bound prostasin had similar molecular weight. The results indicated that prostasin is a true membrane-anchored protein.

Membrane Prostasin Is GPI-anchored

A comparison of the potential carboxyl-terminal membrane-anchorage domain of prostasin (2) to GPI-anchored proteins (28) predicts a GPI linkage for prostasin as well (data not shown). Such a linkage may be susceptible to cleavage by the following reagents: PI-PLC, GPI-PLD (GPI-specific phospholipase D), or nitrous acid (25). In our studies, we first chose PI-PLC to test if prostasin is a GPI-anchored membrane protein. The 293/Pro cells were lysed in TBS containing 1% Triton X-114. After phase separation, the aqueous phase (Figure 5, lane 1, 30 μg total protein) and the detergent phase (lane 2, 3 μg total protein) were analyzed by

western blot using the prostasin-specific antibody. The majority of the prostasin protein in 293/Pro cells is associated with the membrane, which was retained in the detergent phase. The size difference between the soluble and the membrane-bound prostasin may be attributed to the GPI moiety that is linked to prostasin (Figure 5, lanes 1 and 2). The detergent phase was then subjected to PI-PLC digestion at various enzyme concentrations followed by a second phase separation. In Figure 5, lanes 3-5 represent samples of the aqueous phases after PI-PLC digestion. The PI-PLC treatment released prostasin from the detergent phase in a dose-dependent manner (lane 3, 0.25 unit; lane 4, 0.125 unit, and lane 5, zero unit). The PI-PLC-released prostasin and the soluble prostasin are similar in molecular weight as shown in Figure 5. The results support a GPI anchoring mechanism for the membrane-bound prostasin.

One question that remained unclear was whether the native prostasin in the prostate tissue epithelial cells is membrane-bound *via* GPI-anchorage as well. We selected a panel of prostate cancer cell lines and 293/Pro cells that express either recombinant or endogenous prostasin, and normal human prostate tissues in our next experiment. Cell lines that express recombinant prostasin were 293/Pro, PC-3/Pro and DU-145/Pro. The human prostate cancer cell line LNCaP and normal human prostate tissues were used for testing native cellular prostasin. All samples (300 µg of total protein as the starting quantity) were subjected to detergent phase separation before and after PI-PLC digestion as described in Experimental Procedures. PC-3 transfected with a vector plasmid (PC-3/Vec) was used as negative control. The results are presented in Figure 6A. Without PI-PLC treatment, both the recombinant and native prostasin are mainly membrane-anchored (found in the detergent phase). Soluble prostasin is detected in 293/Pro and prostate tissues. After PI-PLC treatment, the membrane-anchored prostasin is released into the soluble fraction from 293/Pro, PC-3/Pro, and prostate tissues, but not from DU-145/Pro and LNCaP. The results indicated that the native prostasin in normal human prostate tissue is also GPI-anchored. The membrane-bound prostasin in DU-145/Pro and LNCaP is resistant to PI-PLC digestion. We further tested if prostasin can be biosynthetically labeled with [^{3}H]ethanolamine, which is specifically incorporated in the GPI unit of GPI-anchored proteins (29). We chose the PC-3/Pro (expressing recombinant prostasin) and LNCaP (expressing native prostasin) cells for [^{3}H]ethanolamine biosynthetic labeling. As shown in Figure 6B, [^{3}H]ethanolamine was incorporated into either recombinant or native prostasin, demonstrating that both are truly GPI-anchored.

13

Identification of A Prostasin-binding Protein

An incubation of the purified r-hPro with mouse or human seminal vesicle fluid yielded a higher molecular weight form of prostasin-containing band as analyzed by SDS-PAGE and immunoblotting. The result presented in Figure 7A indicated that the purified r-hPro formed an 82-kDa complex with a mouse seminal vesicle protein (named as the mouse prostasin-binding protein, or mPBP). The complex was apparently covalently linked, and not *via* a disulfide bond since it was SDS- and heat-stable, and resistant to ß-mercaptoethanol. The complex formation was detected at as early as 1 min post-incubation with a $t_{1/2}$ of ~5 minutes and reached a plateau at ~20 minutes. Densitometry measurements of the complex bands in different lanes were performed using the *LabWork 3.0* Software (Ultra-Violet Products, Upland, CA) (data not shown). Mouse plasma and various tissue extracts including the prostate, coagulating gland, testis, epididymis, vas deferens, adrenal gland, pituitary, thymus, liver, lung, kidney, spleen, heart, brain, uterus, pancreas, and salivary glands; were subjected to the same prostasin-binding assay procedures. No SDS- and heat-stable complex was detected under the same experimental conditions (Figure 7B). A prostasin-binding protein in human seminal vesicle fluid was also identified. As shown in Figure 7C, lane 2, a higher molecular weight complex (82 kDa) was detected after an incubation of r-hPro with the human seminal vesicle fluid. The complex formation between prostasin and the human seminal vesicle prostasin-binding protein was inhibited by heparin (Figure 7C, lane 3). Incubation of mouse or human plasma with r-hPro did not result in formation of any SDS- and heat-stable complex (Figure 7D, lane 1, mouse plasma; lane 3, human plasma). In control assays, mouse plasma was incubated with prostasin for 30 min before seminal vesicle fluid was added for another 30 min of incubation, to demonstrate the binding activity of the prostasin being tested in the presence of plasma (Figure 7D, lane 2). Or, human plasma was incubated with purified human tissue kallikrein and subjected to a western blot analysis using a human kallikrein-specific antibody (30) (Figure 7D, lane 4). The control binding assay showed a 92-kDa kallikrein-kallistatin complex as previously described (16), demonstrating the quality of the human plasma being tested.

The complex formation between prostasin and the mPBP was further investigated by incubating the purified r-hPro with serine protease inhibitors (Figure 8, lanes 2-5), or the prostasin antibody (lane 7 & 8) for 15 min at room temperature before an incubation with mouse seminal vesicle fluid for another 60 min at 37°C. Or, mouse seminal vesicle fluid was first incubated with heparin before the addition of prostasin (Figure 8, lane 6). The complex formation between prostasin and the mPBP was inhibited by serine protease inhibitors, such as

14

aprotinin at dosages of 1 µg/ml and 5 µg/ml, and PMSF at dosages of 1 mM and 5 mM, and by the prostasin antibody (0.1 µl and 0.5 µl). The amount of complex was either reduced or absent in the corresponding lanes of Figure 8. Heparin (1 unit, lane 6) inhibited the complex formation. The complex formation between prostasin and the mPBP without additional reagents was used as the binding reaction control (Figure 8, lane 1). The results suggested that the mPBP interacts with prostasin at the serine active site, and that heparin may alter mPBP's binding property. The properties displayed by the mPBP are shared by the serpin-class serine protease inhibitors. We have observed similar properties for the serpin, kallistatin (16, 17). The predicated molecular mass of the PBP (mouse or human) is estimated at ~47 kDa, given the 40-kDa apparent molecular mass of prostasin, and considering the fact that serpin molecules lose a carboxyl terminal fragment of ~5 kDa when complexed with a serine protease (31).

mPBP Inhibits the Serine Protease Activity of Prostasin

We performed a membrane-overlay zymography analysis to test if the mPBP inhibits prostasin's activity *in vitro*. The prostasin-binding assay was carried out by incubating the purified r-hPro with mouse seminal vesicle fluid in the absence of the serine protease inhibitor aprotinin (Figure 9, lane 3) or in the presence of aprotinin (lane 4). Each sample was then divided into 2 equal portions and subjected to a native PAGE analysis (*i.e.*, SDS and β-mercaptoethanol were not included in the gel solution nor the samples, and the samples were not heated before loading) followed by membrane-overlay zymography (left panel), or western blot analysis using the prostasin antibody (right panel). Mouse seminal vesicle fluid proteins alone (Figure 9, lane 1) displayed no enzymatic activities toward the synthetic substrate D-Pro-Phe-Arg-AFC (left panel), nor cross-reactivity with the prostasin antibody (right panel). The purified r-hPro alone (lane 2) demonstrated enzymatic activity toward D-Pro-Phe-Arg-AFC (left panel) and was recognized by the prostasin antibody (right panel). When prostasin formed a complex with mPBP in the mouse seminal vesicle fluid, it no longer cleaves D-Pro-Phe-Arg-AFC since no fluorescence is present at the complex band location in lane 3 of the left panel, while the complex is identified by the prostasin antibody as the upper band in lane 3 of the right panel. The remaining unbound prostasin yielded, expectedly, lesser fluorescence (left panel, lower band in lane 3, as compared to lane 2) and was recognized by the prostasin antibody (right panel, lane 3). When the purified r-hPro was pre-incubated with the serine protease inhibitor aprotinin before incubation with the mouse seminal vesicle fluid, no complex was detected (lane 4, right panel). A reduced level of fluorescence appeared at the prostasin band in lane 4 of the

left panel, due to the presence of aprotinin. Since the binding of aprotinin to prostasin was reversible while proteins were being resolved in the gel, the inhibition of prostasin activity seen in lane 4 was not complete. The results suggested that mPBP not only binds to prostasin at the serine active site but also inhibits prostasin's serine protease activity *in vitro*. Two bands were observed in the prostasin alone sample in the immunoblot (Figure 9, right panel, lane 2), the differential mobility may be caused by differential glycosylation (1). Aprotinin binding to prostasin changes the charge/mass ratio of the protein, therefore, migration of the aprotinin-bound prostasin in a native PAGE could change as well, potentially causing the multiple-banding pattern seen in lane 4 of the immunoblot (Figure 9, right panel).

Membrane-Prostasin Binds to mPBP

To test if the membrane-bound prostasin has binding activity toward the mPBP, the 293/Pro and PC-3/Pro cells were subjected to differential centrifugation as described in Experimental Procedures except that no protease inhibitors were added during membrane fractionation. Immediately after centrifugation, an aliquot of each membrane fraction (30-40 µg of total protein) was incubated with an aliquot of mouse seminal vesicle fluid (5 µl) at 37°C for 1 hr. The binding mixture was then analyzed by western blot analysis using a prostasin-specific antibody. In Figure 10, the left panel shows that the membrane-bound prostasin in 293/Pro cells (P2 and P3 fractions) formed an 82-kDa complex when incubated with mouse seminal vesicle fluid. The prostasin protein in the crude nuclear fraction (P1) and the cytosol (S) did not form any detectable complex. The purified r-hPro from the conditioned medium (*i.e.*, the secreted prostasin) was used as positive control for the binding assay. In Figure 10, right panel, we showed that the membrane-bound prostasin in PC-3/Pro cells also formed a complex with mPBP and this reaction was inhibited by the serine protease inhibitor aprotinin.

Discussion

The prostasin serine protease is predominantly synthesized in the prostate in human (1). Recently our laboratory demonstrated that prostasin expression is significantly down-regulated in high-grade prostate tumors and absent in invasive human and mouse prostate cancer cell lines. We have also shown, in an *in vitro* Matrigel invasion assay, that cellular prostasin may be an invasion suppressor of prostate cancer (20). In the present study, we intended to investigate prostasin's intracellular distribution and to determine whether the cellular prostasin is an active serine protease, to provide clues to the potential mechanisms of prostasin's cellular function.

We first established a mammalian expression system to produce a recombinant human prostasin. The purified secreted recombinant prostasin displayed biochemical characteristics similar to that of the native prostasin (purified from human semen, Ref. 1), such as the molecular weight on SDS-PAGE, immunological reactivity (Figure 1), enzymatic activity toward the synthetic substrate D-Pro-Phe-Arg-AFC (Figure 9), and responsiveness to serine protease inhibitors (Figures 8 and 9). We then used a polyclonal antibody specific for human prostasin to determine whether prostasin can exist in a membrane-bound form because its predicted structure suggested this possibility (2). By means of sequential centrifugation of the 293/Pro and PC-3/Pro cell components (as shown in Figure 2 & 3A), we were able to identify prostasin in various subcellular compartments, such as the crude nuclei, heavy membranes (including mitochondria, lysosomes, and peroxisomes), and light membranes (including plasma membrane, microsomes, and endoplasmic reticula). A confocal microscopy analysis of the PC-3/Pro cells (Figure 3B) revealed prostasin's subcellular localization to be primarily at the nuclear/ER/Golgi complex (27). The immunofluorescently localized prostasin at the nuclear/ER/Golgi complex is believed to be that identified in the western blot analysis of the nuclear fraction P1. The endogenously expressed prostasin in the LNCaP cells, however, was only detected in the light membrane fraction P3 (Figure 3A), and was not detected at the nuclear/ER/Golgi complex (Figure 3B). The different subcellular localization of prostasin between the recombinant expression system and the endogenous expression system may be caused by expression level differences. Cells expressing recombinant prostasin produced high amounts of prostasin with the 293/Pro being the highest followed by PC-3/Pro and DU-145/Pro. The prostasin expression level in the LNCaP cells was considerably lower than that in these transfected cell lines. The expression levels were determined by a semi-quantitative western blot analysis (data not shown). Alternatively, different cell lines may have different protein sorting mechanisms, leading to different subcellular localization patterns (32). On the other

hand, GPI-anchored prostasin might be associated with sterols, and therefore, can be found in many compartments of the cell including the plasma membrane, the Golgi apparatus, ER, nucleus, lysosomes and mitochondria, and in lipid particles (33). Prostasin in the nuclear fraction (P1) did not show binding activity to the prostasin-binding protein (Figure 10). It is presently unclear why prostasin in this fraction was unable to form a complex with the mPBP. The functional significance of prostasin in the nuclear/ER/Golgi complex is also unclear at present and will be investigated in the future.

Despite the apparently different subcellular localization of prostasin in overexpressing cells versus endogenously expressing cells, prostasin is found in an membrane-bound form in all cell lines tested, as well as in normal human prostate tissues (Figure 6A). The membrane-bound prostasin was released when extracted with a detergent, but remained membrane-bound when treated with high salt or alkali (Figure 4), ruling out the possibility that prostasin is associated with another membrane-bound protein *via* non-covalent linkages. We also demonstrated that the membrane-bound and the detergent-released prostasin have similar molecular mass (Figure 4), ruling out the possibility that prostasin is covalently linked to another membrane-bound protein.

The native prostasin in normal prostate tissue and the recombinant prostasin in 293/Pro and PC-3/Pro cells were easily released from the membrane with PI-PLC treatment (Figures 5 and 6A), suggesting that prostasin is bound to the membrane *via* a GPI-anchor rather than through a true transmembrane domain. The membrane-bound prostasin in LNCaP cells (native) or DU-145/Pro cells (recombinant), however, was resistant to PI-PLC treatment (Figure 6A). As reported in Englund (34) and Hiroshi *et al.* (35), not all GPI-anchored proteins are susceptible to PI-PLC digestion. The membrane-anchored prostasin in LNCaP and DU-145/Pro could potentially be susceptible to other phospholipases such as GPI-PLD (34, 35). Our results from the [^{3}H]ethanolamine biosynthetic labeling experiment with PC-3/Pro and LNCaP cells, however, provided direct evidence that in the prostate epithelial cells recombinant or native prostasin is GPI-anchored, regardless of its sensitivity to PI-PLC treatment (Figure 6B).

Among all four human cell lines that express either recombinant or native prostasin, as well as normal human prostate tissue, prostasin exists mainly as a membrane-bound protein (Figure 6A). A small portion of prostasin in the 293/Pro cells is in the cytosolic fraction. This cytosolic prostasin could be a mis-processed or mis-folded form that was exported from the ER before GPI-anchor attachment, a mechanism previously documented (36). The presumably mis-folded prostasin in the cytosol had no binding activities when it was incubated with mouse

seminal vesicle fluid, possibly due to the mis-folding. The secreted recombinant prostasin, when purified from the 293/Pro culture media, however, is enzymatically active and able to form a complex with the mPBP (Figures 9-10), indicating that the cytosolic prostasin is not the source of secreted prostasin. The soluble fraction of prostasin seen in the human prostate tissues (Figure 6A) before PI-PLC treatment may also be a mis-folded form by the same mechanism described above or may be attributed to residual prostatic fluid due to possible incomplete washing before tissue lysis.

We identified a prostasin-binding protein (PBP) in mouse and human seminal vesicles (Figure 7). Prostasin forms an 82-kDa, SDS- and heat-stable complex when incubated with seminal vesicle fluid as determined by SDS-PAGE under reducing conditions followed by prostasin immunoblotting. This complex is apparently covalently formed between prostasin and the PBP, and not *via* a disulfide linkage. We have chosen to use mouse seminal vesicles for an in-depth analysis of the PBP because of easier availability. The complex formation between prostasin and the mPBP was inhibited by the polyclonal prostasin antibody, heparin, and serine protease inhibitors. In a membrane-overlay zymography analysis (Figure 9), the prostasin-mPBP complex showed no activities to a synthetic substrate D-Pro-Phe-Arg-AFC, while unbound prostasin was active. These results suggest that mPBP may be a serpin-class serine protease inhibitor. The true nature of the mechanism of prostasin inhibition by the mPBP will be investigated upon purification and sequence analysis of this protein. An incubation of mouse or human plasma with r-hPro did not result in formation of any covalently-bound complex. This result would rule out the possibility of the mPBP being one of the known members of the serpin family present normally in the blood, such as α1-antitrypsin, α1-antichymotrypsin, kallistatin, plasminogen activator inhibitor (PAI), and protein C inhibitor. At present, the functional significance of the prostasin-binding protein with respect to prostate biology is unclear. Future studies will be aimed at determining the prostasin-binding site in the PBP, which could potentially reveal clues on prostasin's natural protein substrate.

One of our goals for the present study was to determine whether the membrane-anchored prostasin is an active serine protease. To accomplish this, we needed a prostasin-specific enzymatic activity assay that is applicable for membrane-bound prostasin since this form of prostasin exists in a complex mixture. The membrane-overlay zymography assay was not applicable for the membrane-anchored prostasin since lipid-associated proteins can not be well resolved in non-denaturing native gel electrophoresis. The identification of the mPBP offered us an indirect but prostasin-specific assay to address this question. As presented in

Figure 10, the membrane-bound human prostasin also displayed binding activity to the mPBP, and the binding is inhibited by a serine protease inhibitor (aprotinin) that competes for the serine active site, suggesting that the membrane-bound prostasin is likely an active serine protease. Demonstration of membrane-bound prostasin being an active serine protease will provide clues for investigating the signal transduction pathway(s) involved in the anti-invasion activity of prostasin since this anti-invasion activity is conferred by the cellular prostasin, but not the secreted prostasin (20).

Prostasin is made in the prostate and secreted as an active serine protease (1) while the prostasin-binding protein is made in the seminal vesicles. The fact that prostasin forms a complex with the PBP suggests that the two proteins interact with each other when semen is ejaculated, thereby, implicating a role for both proteins in semen coagulation and liquefaction. Prostasin and the prostasin-binding protein in male reproductive tracts may serve together in a partnership to affect fertility. Investigating the prostasin/prostasin-binding protein partnership could also lead to a better understanding of the various factors affecting fertility or, causing infertility. Overall, prostasin, as a GPI-anchored, or a secreted active serine protease, may have multiple physiological functions, depending on the localization of the prostasin protein, whether it is membrane-bound or secreted.

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Figure Legends:

Figure 1. SDS-PAGE and western blot analysis. Recombinant human prostasin purified from serum-free conditioned medium of the 293/Pro cells was analyzed on a 10% SDS-PAGE under reducing conditions. The purified recombinant prostasin (3 µg) migrates at 40 kDa (left panel, r-hPro), and is recognized by an antibody made against the purified recombinant prostasin (IB: r-Pro Ab, lane 1) as well as a prostasin-specific antibody made against purified native prostasin (IB: n-Pro Ab, lane 1, Ref. 1). The quantity of purified prostasin in lanes 1 is 0.5 µg. Samples from the 293/Pro cell lysate (lanes 2, 20 µg), 293/Vec (lanes 3, 20 µg) cell lysate, and human semen (lanes 4, 30 µg) were immunoblotted with r-Pro Ab (upper right panel, 1:1,000 dilution) and n-Pro Ab (lower right panel, 1:1,000 dilution). Both antibodies recognize the recombinant prostasin as well as the native prostasin but do not have cross-reactivity with 293/Vec proteins.

Figure 2. Analysis of prostasin in 293/Pro cell fractions. The cells were fractionated through differential centrifugation. An equal amount of protein (30 µg) from each centrifugation step was resolved on a 10% SDS-PAGE followed by immunoblotting with a prostasin-specific polyclonal antibody (1:1,000 dilution). Prostasin (40 kDa) is detected in the nuclear fraction (P1), heavy-membrane fraction (P2, including mitochondria, lysosomes, and peroxisomes), light-membrane fraction (P3, including plasma membrane, microsomes, and endoplasmic reticula) as well as the cytosol (S) of 293/Pro. Purified r-hPro (0.5 µg) was used as positive control.

Figure 3. Analysis of prostasin in prostate cancer cell lines. A: Western blot analysis of membrane-bound prostasin in PC-3/Pro and LNCaP cells. The experimental procedures were the same as described in Figure 2. Prostasin (40 kDa) is detected in the nuclear fraction (P1), heavy-membrane fraction (P2), light-membrane fraction (P3), but not in the cytosol (S) of PC-3/Pro. In LNCaP cell fractions, prostasin is detected only in P3. Antibodies against a nuclear protein, the PARP (1:500, or 0.5 ng/ml), and a plasma membrane protein, β1-integrin (1:1,000, or 0.25 ng/ml), were used as fractionation markers. B: Confocal microscopic localization of prostasin. The PC-3/Pro and LNCaP cells were fixed, permeablized, and subjected to a double-immunostaining. One focal plane for each cell type is presented to show prostasin signals (green). Prostasin is primarily detected at the nuclear/ER/Golgi complex as well as punctate regions in the PC-3/Pro cell. In the LNCaP cells, no punctate prostasin can be seen. The nuclear marker protein PARP (red) is seen in both cell types. A merge image for either cell type

is presented to the right. The images were taken after subtracting background signal on a pre-immune serum-stained control coverslip. Magnification: 400x. The antibody dilution ratios are: anti-prostasin, 1:100; anti-PARP, 1:75 (or 3.3 ng/ml); goat anti-rabbit IgG-FITC, 1:50; and goat anti-mouse IgG-Cy3, 1:800.

Figure 4. Recombinant prostasin is a true membrane-bound protein. Approximately 80-100 μg total protein of each membrane fraction of PC-3/Pro cells (as described in legend to Figure 3A) were subjected to detergent (TX), high salt (HiS) or alkali (Alk) treatment followed by centrifugation to separate the supernatant and the pellet for SDS-PAGE and western blot analysis. Prostasin in all fractions can only be released from the membrane (pellet) to the supernatant (soluble protein) by the detergent treatment, but not high salt or alkali treatment.

Figure 5. Detergent phase separation of 293/Pro cells and phospholipase C treatment. The 293/Pro cells were lysed in TBS containing 1% Triton X-114 and subjected to phase separation (700-800 μg of total protein as the starting quantity). The detergent phase containing membrane-associated proteins (equivalent to 1/10 of the total starting membrane-associated proteins) was further treated with phosphatidylinositol-specific phospholipase C (PI-PLC) followed by additional phase separation. The soluble proteins (lane 1, 30 μg) and the detergent phase proteins (lane 2, 3 μg) before PI-PLC treatment, and the soluble proteins extracted from the detergent phase after PI-PLC treatment were subjected to SDS-PAGE and western blot analysis using a prostasin-specific antibody. The membrane-bound prostasin is released from the membrane after PI-PLC digestion as it was detected in the post-PLC soluble phase. The amounts of PI-PLC used in the reactions are: lane 3, 0.25 unit; lane 4, 0.125 unit, and lane 5, zero unit in a total reaction volume of 100 μl. The results indicate that prostasin is anchored to membrane *via* GPI. The size difference between the membrane-bound prostasin and the soluble prostasin (lanes 1 and 2) may be attributed to the GPI moiety that is linked to prostasin.

Figure 6. Prostasin is a GPI-anchored membrane protein. A: Detergent phase-separation and PI-PLC treatment. Human prostate tissues and cell lines that express either endogenous prostasin (LNCaP) or recombinant prostasin (293/Pro, PC-3/Pro, and DU-145/Pro) were subjected to detergent phase separation/PI-PLC digestion followed by prostasin immunoblotting

(300 µg of total protein were used for each sample as the starting quantity). Both the recombinant and native prostasin are mainly membrane-anchored (pre-PLC, detergent phase or D). Soluble prostasin is detected in 293/Pro and prostate tissues (pre-PLC, soluble phase or S). After PI-PLC treatment, the membrane-anchored prostasin is released into the soluble fraction from 293/Pro, PC-3/Pro, and prostate tissues (post-PLC, S), but not from DU-145/Pro and LNCaP (post-PLC, D). The PC-3/Vec cells showed negative results in all fractions tested. Due to a high-level prostasin expression in the 293/Pro cells, a portion of prostasin remained in the detergent phase after PI-PLC digestion. B: Incorporation of [³H]ethanolamine into prostasin. PC-3/Pro or LNCaP cells were incubated with [³H]ethanolamine (100 µCi) in 1 ml of OPTI-MEM I serum-free medium for 24 h in 5% CO$_2$ at 37°C. The cell lysate were subjected to immunoprecipitation using the prostasin antibody (purified IgG fraction, 2 µg/ml) and Protein A Sepharose beads as described in "Experimental Procedures." After SDS-PAGE separation of the samples, the labeled protein was detected by fluorography using Amplify fluorographic reagent and exposure to an X-ray film at –80°C with an intensifying screen for 14 days.

Figure 7. Complex formation between prostasin and its binding protein. A: Purified recombinant prostasin (0.5 µg) was incubated with mouse seminal vesicle fluid at 37°C for various time periods as indicated. The samples were subjected to an SDS-PAGE under reducing conditions followed by immunoblotting with a prostasin-specific antibody. Prostasin forms an 82-kDa complex (upper arrow) with the mPBP in mouse seminal vesicle fluid. The complex formation can be detected at as early as 1 min post-incubation. Excess unbound prostasin is indicated by the lower arrow. Prostasin alone without incubation with mouse seminal vesicle fluid was labeled as "0" min. B: Various mouse tissue extracts were analyzed in a prostasin-binding assay and prostasin immunoblotting as described. SVF: mouse seminal vesicle fluid, Coag. gl.: coagulating gland, Vas def.: vas deferens, Adrenal gl.: adrenal gland, Salivary gl.: salivary glands. C: Purified recombinant prostasin (0.5 µg) was incubated with human seminal vesicle fluid at 37°C for 60 min. Similarly, prostasin forms an 82-kDa complex with the hPBP in human seminal vesicle fluid (lane 2) and the complex formation was inhibited by heparin (1 unit, lane 3). The purified recombinant prostasin alone was used as control (lane 1). D: Human or mouse plasma was tested in prostasin binding assay. HUK: human urinary (tissue) kallikrein (0.5 µg), Pro: purified recombinant prostasin (0.5 µg), SVF: mouse seminal vesicle fluid (5 µl), MP: mouse plasma (1 µl), HP: human plasma (1 µl). Lanes 1-3: probed with prostasin antibody (1:1,000); and lane 4: probed with a human kallikrein antibody (1:1,000).

26

Figure 8. Inhibition of complex formation between prostasin and mPBP. Purified recombinant prostasin (0.5 μg) was incubated with mouse seminal vesicle fluid (5 μl) in the present of aprotinin (lanes 2 & 3), PMSF (lanes 4 & 5), heparin (lane 6), prostasin antibody (lanes 7 & 8) at 37°C for 1 hr. All samples were subjected to SDS-PAGE under reducing conditions followed by immunoblotting with a prostasin-specific antibody. The complex formation (upper arrow) between prostasin and the mPBP in mouse seminal vesicle fluid is inhibited by aprotinin, PMSF, heparin, and the antibody against prostasin. The "*" indicates the IgG heavy chain and light chain recognized by the goat anti-rabbit secondary antibody used in the western blot analysis. Excess unbound prostasin is indicated by the lower arrow. Complex formation between r-hPro and the mPBP in mouse seminal vesicle fluid without any other reagent was used as positive control (lane 1).

Figure 9. Membrane-overlay zymography. Samples from a prostasin-binding assay were resolved on a 10% native acrylamide gel without SDS/boiling or β-mercaptoethanol. The gel was then either overlaid with a membrane impregnated with a prostasin substrate (D-Pro-Phe-Arg-AFC) (left panel) or transferred for prostasin immunoblotting (right panel). Lane 1, mouse seminal vesicle fluid alone (5 μl); lane 2, purified r-hPro alone (0.5 μg); lane 3, mixture of r-hPro and mouse seminal vesicle fluid; and lane 4, the same as lane 3 except r-hPro was pre-incubated with aprotinin (5 μg/ml) for 15 min before the addition of mouse seminal vesicle fluid. The fluorogenic substrate impregnated in the membrane was hydrolyzed by prostasin in the gel and iso-prostasin patterns in the membrane appear as fluorescent bands. The results suggested that the mPBP not only binds to prostasin at the serine active site but also inhibits prostasin's serine protease activity *in vitro*.

Figure 10. The membrane-bound prostasin forms a complex with mPBP. Membrane fractions of 293/Pro and PC-3/Pro cells (30-40 μg of total protein) were incubated with mouse seminal vesicle fluid (5 μl) at 37°C for 1 hr. Right panel, samples in lanes 3 & 4 were pre-incubated with aprotinin before addition of mouse seminal vesicle fluid. All sample mixtures were analyzed on an SDS-PAGE under reducing conditions followed by prostasin immunoblotting. P1, nucleus fraction; P2 heavy membranes; P3 light membranes; S, cytosolic proteins; and r-hPro, purified recombinant prostasin. The membrane-bound prostasin in heavy-(P2) or light-membrane fractions (P3) formed a complex (upper arrow) when incubated with
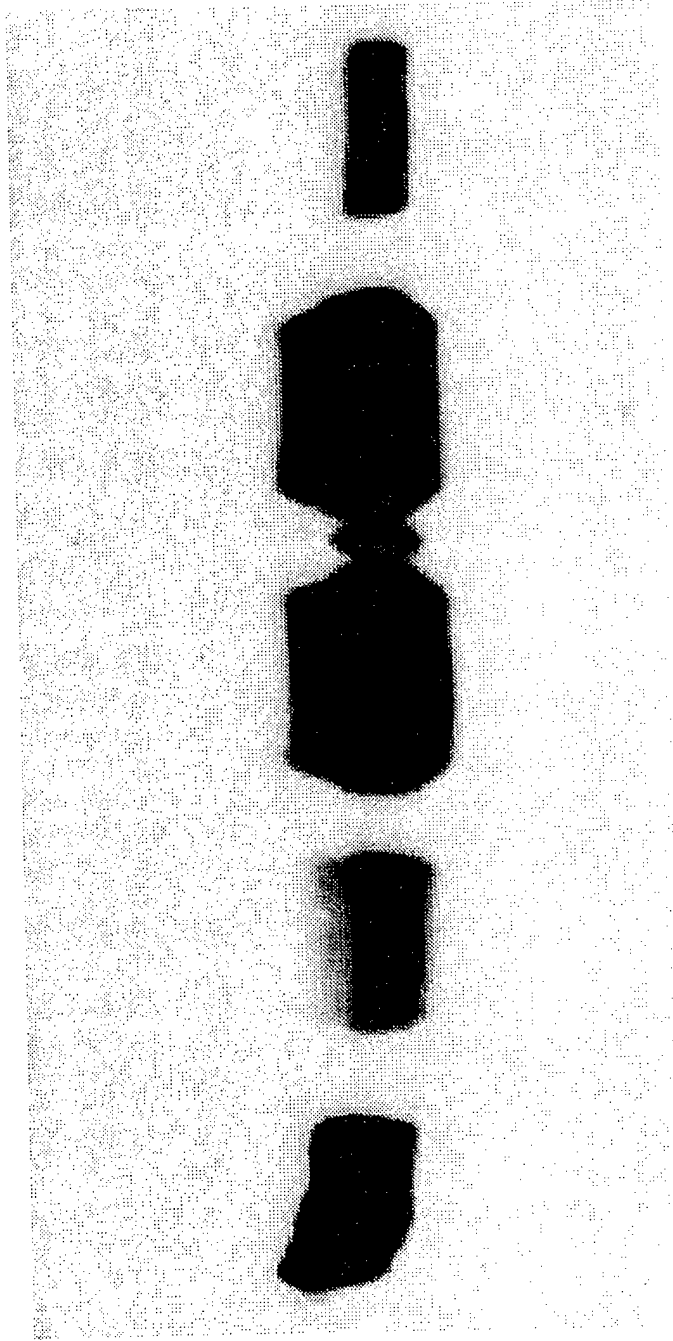
mouse seminal vesicle fluid, while prostasin in P1 and cytosolic fractions showed no complex formation. r-hPro was used as positive control in the *in vitro* binding assay. Excess unbound prostasin is indicated by the lower arrow. Addition of aprotinin at 5 µg/ml inhibited the complex formation as shown in the right panel.
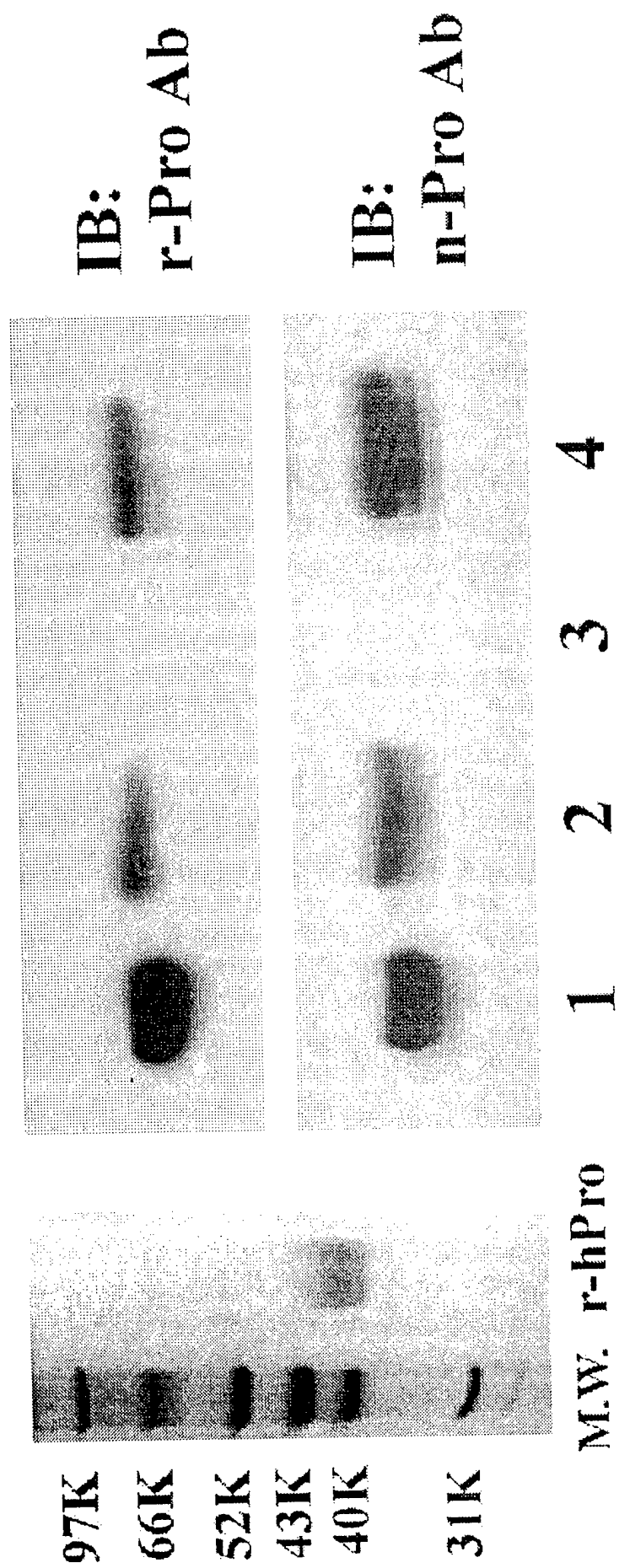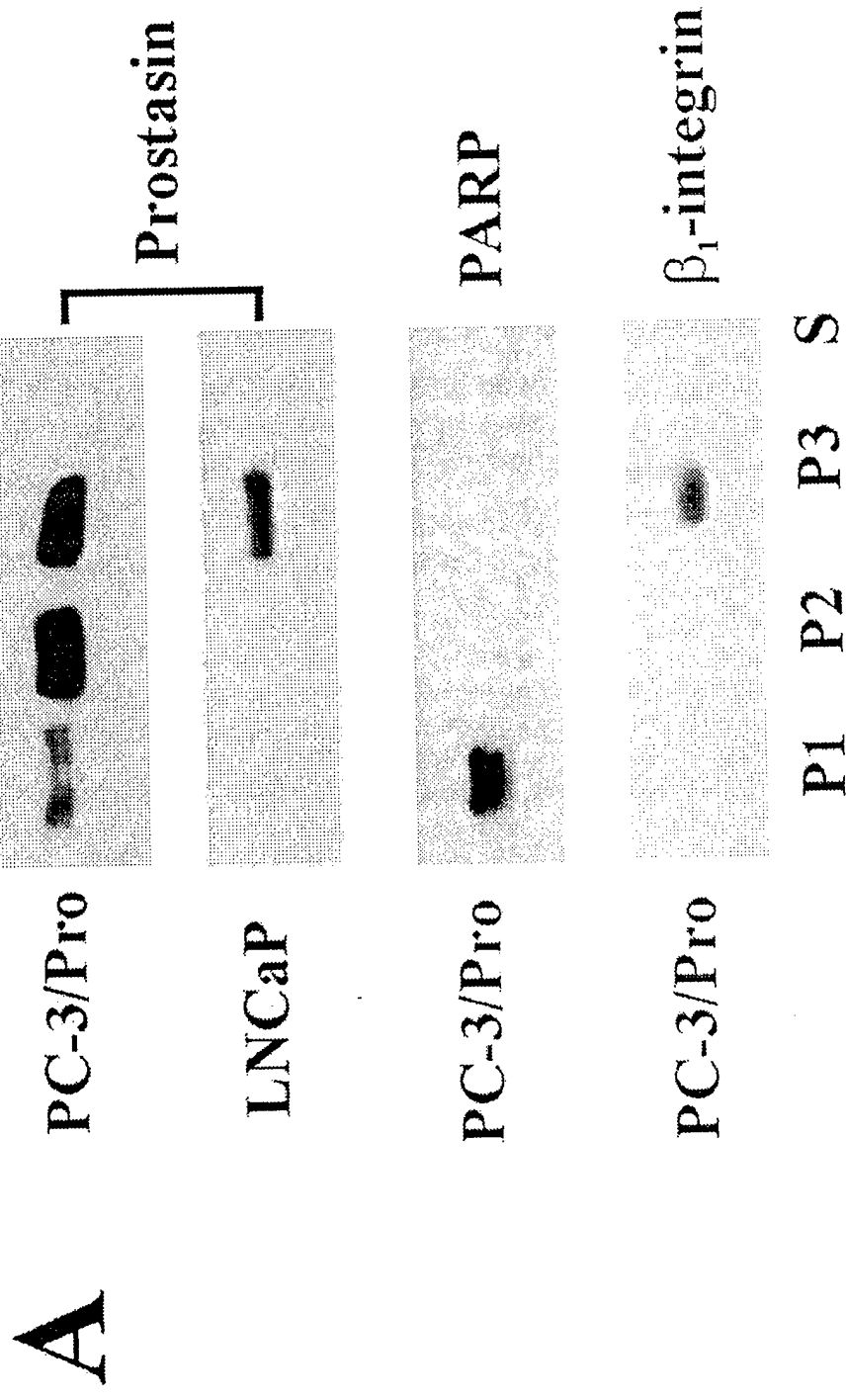
Figure 1

Prostasin
40 kDa

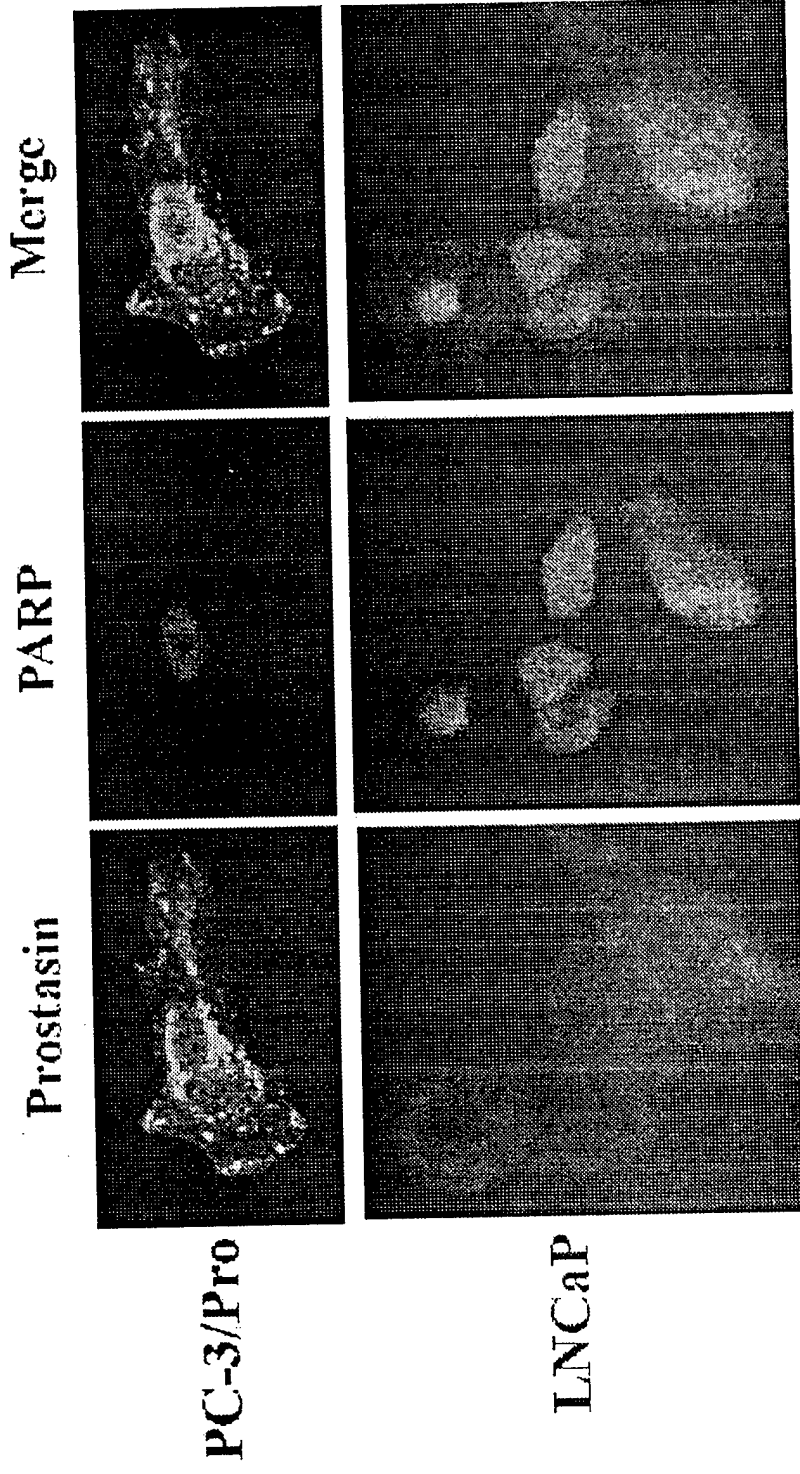r-hPro   P1   P2   P3   S

Figure 2
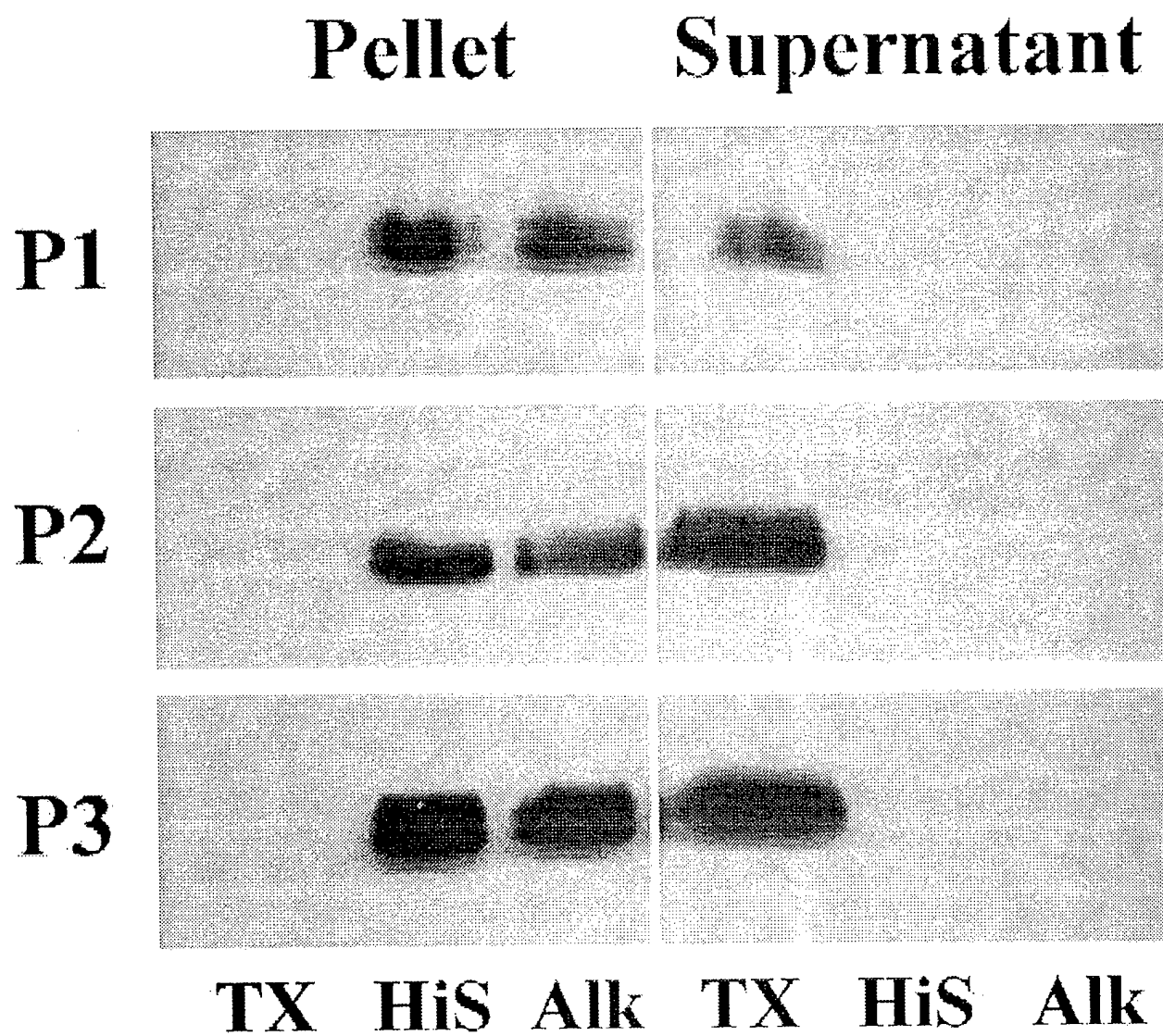
A

Prostasin

PC-3/Pro

LNCaP

PARP

PC-3/Pro

β₁-integrin

PC-3/Pro

P1 P2 P3 S

Figure 3A

B

Prostasin	PARP	Merge

PC-3/Pro

LNCaP

Figure 3B

Figure 4

Figure 5

Figure 6A

B

118 K —

82 K —

50 K —

33 K —

27 K —

PC-3/Pro   LNCaP

Figure 6B

A

Complex 82 kDa →

Prostasin 40 kDa →

0 0.5 1 5 10 20 30 75 90 180 min.

Figure 7A

B

Figure 7B

C

Complex
82 kDa →

Prostasin
40 kDa →

1   2   3
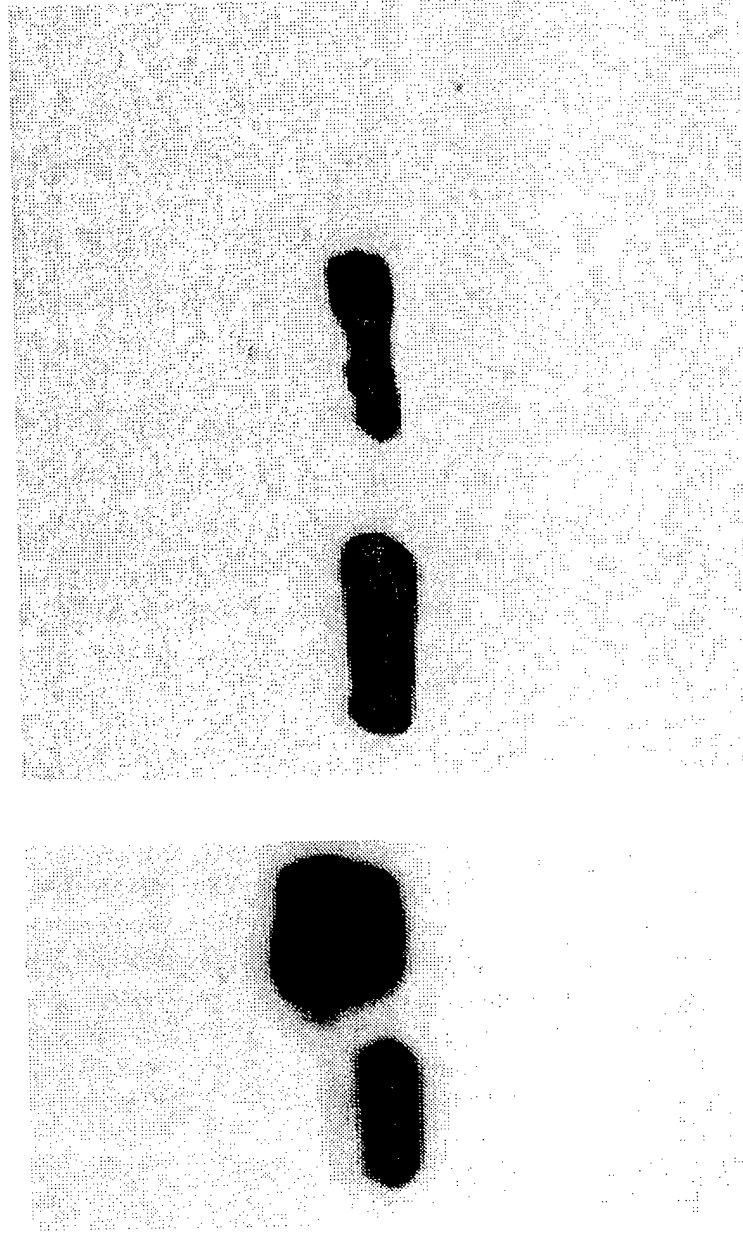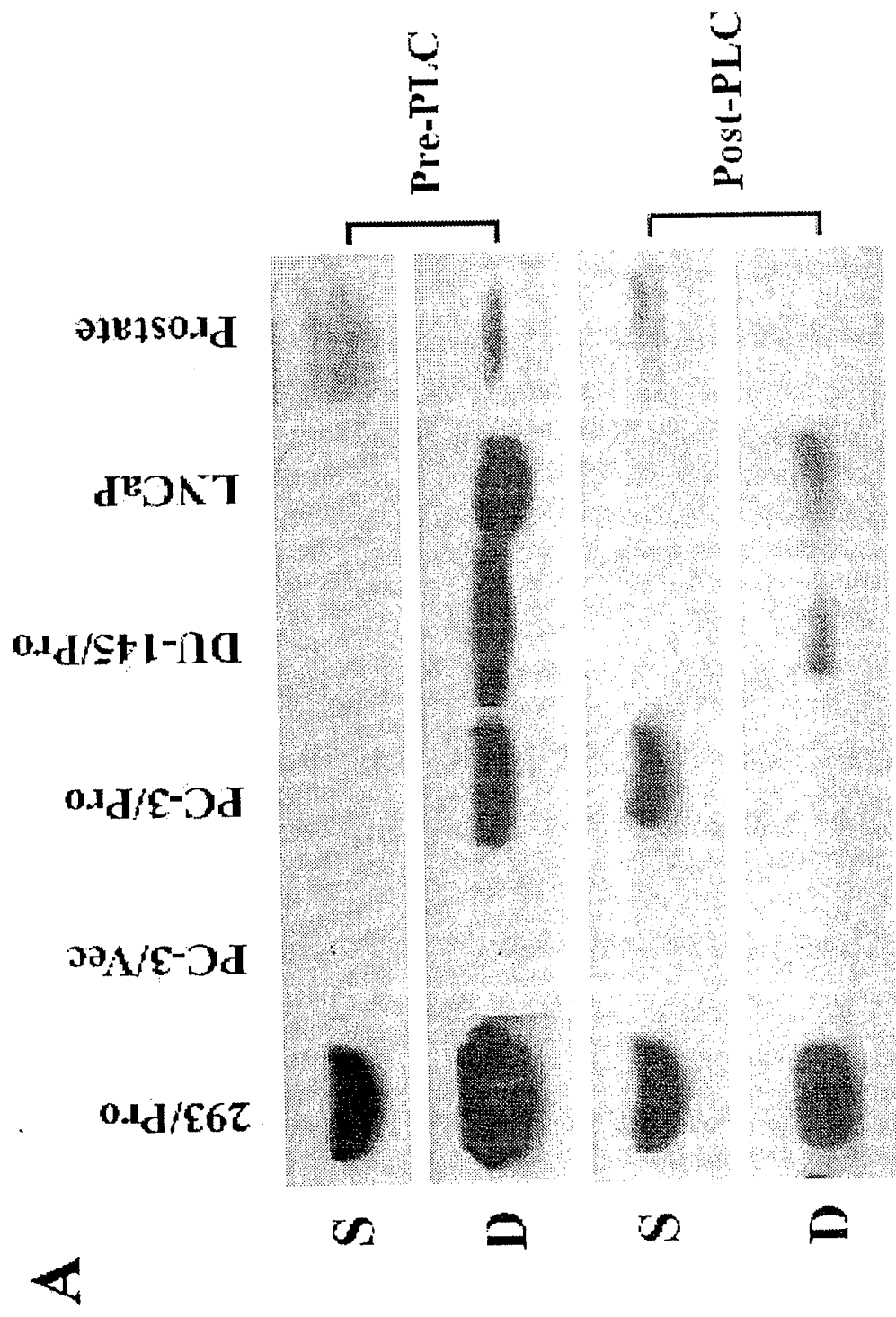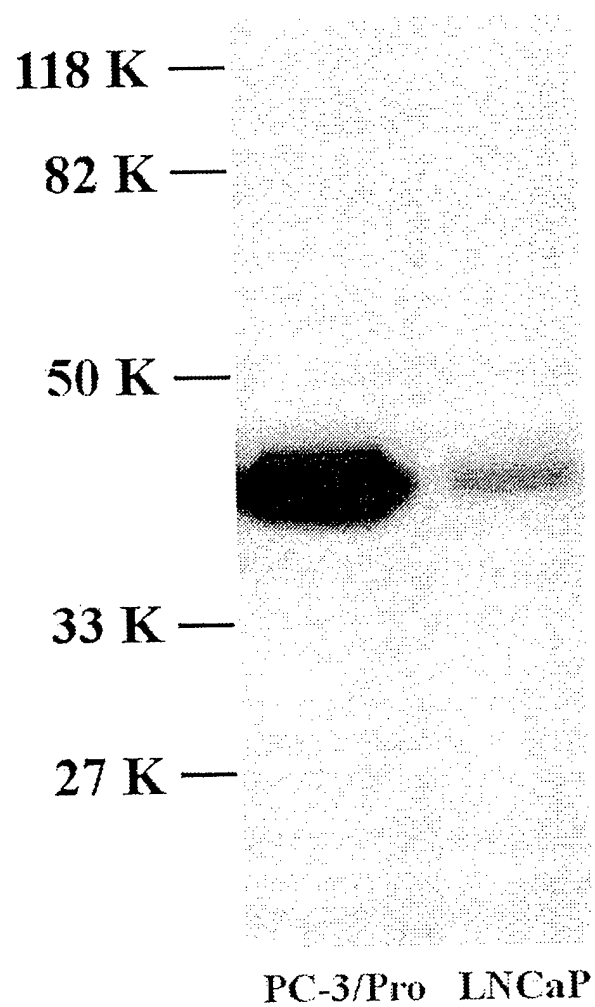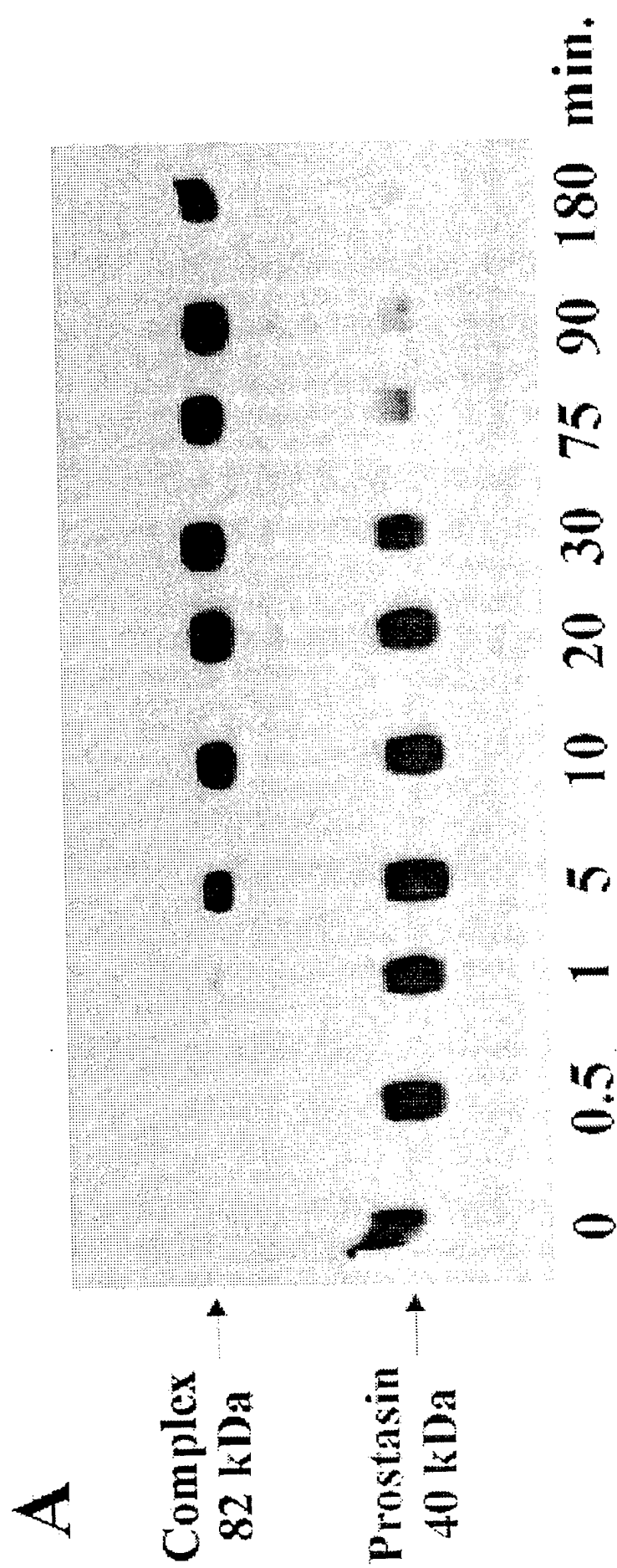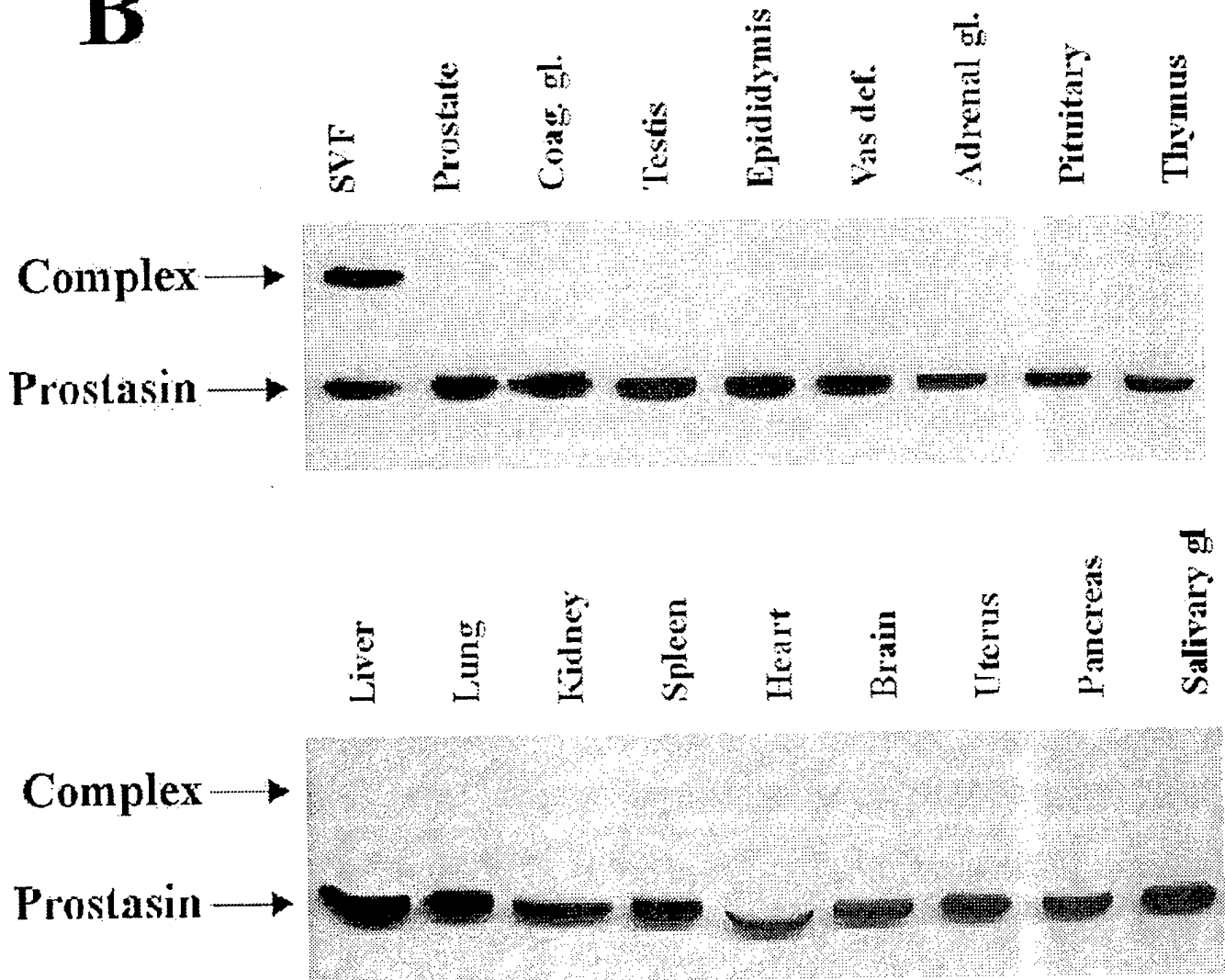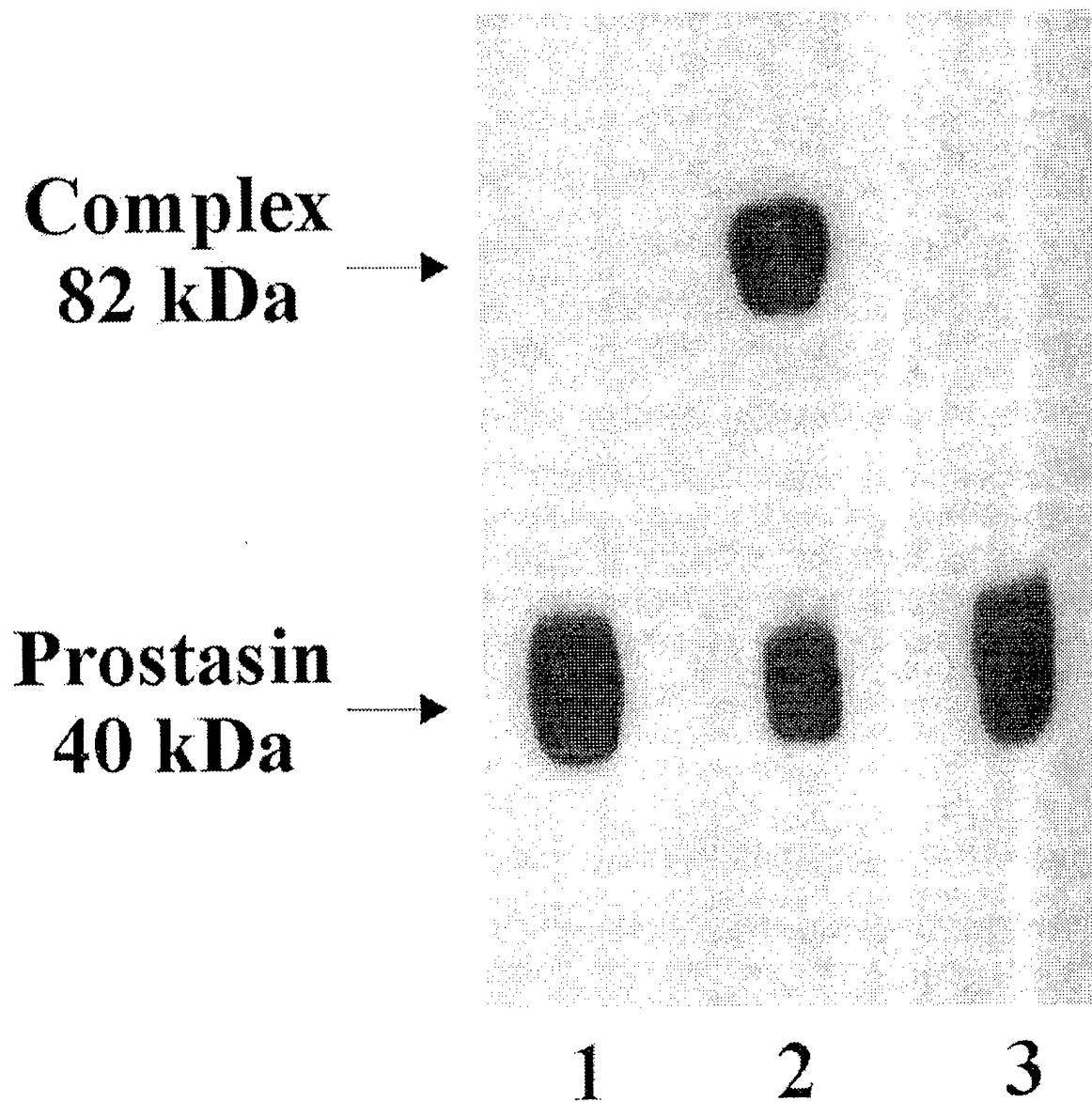
Figure 7C

D

HUK	–	–	–	+
Pro	+	+	+	–
SVF	–	+	–	–
MP	+	+	–	–
HP	–	–	+	+

92kDa
82kDa

1 2 3 4

Figure 7D

Complex ⟶

Prostasin ⟶

1 2 3 4 5 6 7 8

1. No Inhibitors
2. Aprotinin 1μg/ml
3. Aprotinin 5μg/ml
4. PMSF 1mM
5. PMSF 5mM
6. Heparin 1u
7. r-Pro Ab 0.1μl
8. r-Pro Ab 0.5μl
*. IgG (H+L)

Figure 8

Figure 9

Figure 10